# AN INVESTIGATION OF THE PHARMACOLOGY AND UNDERLYING MECHANISM OF THE RESPONSES TO GAMMA-AMINO BUTYRIC ACID AND ETHYLENEDIAMINE OF PYRAMIDAL NEURONES IN THE HIPPOCAMPAL SLICE PREPARATION

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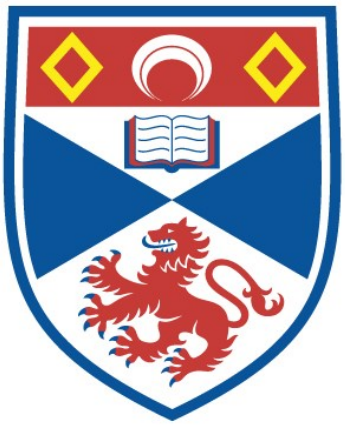

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# AN INVESTIGATION OF THE PHARMACOLOGY AND UNDERLYING MECHANISM OF THE RESPONSES TO GAMMA-AMINO BUTYRIC ACID AND ETHYLENEDIAMINE OF PYRAMIDAL NEURONES IN THE HIPPOCAMPAL SLICE PREPARATION.

by

Timothy J. Blaxter

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I was admitted as a research student under ordinance No. 12 on October 1, 1980 and as a candidate for the degree of Ph.D. on October 1, 1980; the higher study for which this is a record was carried out in the University of St Andrews between 1980 and 1983.

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I, Timothy John Blaxter, hereby certify that this thesis which is approximately 30,000 words in length has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Doctor of Philosophy of the University of St Andrews and that he is qualified to submit this thesis in application for this degree.

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CONTENTS.

SUMMARY

1. Hippocampal pyramidal neurones were successfully maintained *in vitro* in brain slices.

2. Stable intracellular recordings were made from pyramidal neurones in the CA1 region of the pyramidal neurone layer.

3. The iontophoresis of acetylcholine, glutamate, GABA and ethylenediamine produced responses when applied to the dendrites and when applied to the cell body of pyramidal neurones.

4. Ethylenediamine may have acted indirectly on GABA receptors.

5. The depolarizing response of the pyramidal neurone dendrites to GABA was dependent on chloride ions and was also probably dependent on sodium ions.

6. The hyperpolarizing response of the pyramidal neurone dendrites to GABA and ethylenediamine was dependent on potassium ions.

7. The hyperpolarizing response of the pyramidal neurone cell body to GABA and ethylenediamine was dependent on potassium ions.

8. Bicuculline reduced the depolarizing response of the dendrites to GABA and ethylenediamine and reduced the

hyperpolarizing response of the cell body to GABA.

9. Picrotoxin reduced the depolarizing response of the dendrites to GABA and ethylenediamine.

10. The hyperpolarizing response of the dendrites to GABA and ethylenediamine was not mediated by the usual bicuculline- and picrotoxin- sensitive receptor.

11. Benzodiazepines had variable effects on the depolarizing response of the dendrites to GABA or ethylenediamine and variable effects on the hyperpolarizing response of the cell body to GABA or ethylenediamine. Benzodiazepines had no effect on the hyperpolarizing response of the dendrites to EDA.

12. The hyperpolarizing response of the dendrites to GABA and ethylenediamine was distinct from the hyperpolarizing response of the cell body to GABA and ethylenediamine.

## LIST OF ABBREVIATIONS.

GABA.....gamma-amino butyric acid.

EDA.....ethylenediamine dihydrochloride.

SITS.....disodium 4-acetoamido-4´-thiocyanato stilbene-2-2´ disulphonic acid.

THIP.....4,5,6,7, tetrahydroisoxazolo [3-5c]-pyrid-3-ol.

KAc.....potassium acetate.

ACSF.....artificial cerebro-spinal fluid.

DRC.....dose response curve.

LDRC.....log dose-response curve.

$[Cl^-]_o$.....external chloride ion concentration.

$[Cl^-]_i$.....internal chloride ion concentration.

$[Na^+]_o$.....external sodium ion concentration.

$[K^+]_o$.....external potassium ion concentration.

$E_{Cl}$.....equilibrium potential for chloride ions.

$E_{Na}$.....equilibrium potential for sodium ions.

$E_K$.....equilibrium potential for potassium ions.

$E_{rev}$.....reversal potential of the response.

$R_m$.....input resistance of the neurone.

$R_m$*.....input resistance of the neurone in the presence of a drug.

$V_m$.....membrane potential measured on withdrawal of the electrode.

# CHAPTER ONE.

## INTRODUCTION.

This introduction is divided into 3 sections:

1. Status of GABA as a neurotransmitter in crustaceans and in mammals. Crustaceans were chosen because the evidence is most compelling and the major advances have been made in this order.
2. The hippocampus and the status of GABA as a neurotransmitter in the hippocampus.
3. GABA agonists and the benzodiazepines.

During the last 30 years, GABA has become established as a putative neurotransmitter in many animal orders, especially the crustacea and the mammals. It is in these orders that the criteria for neurotransmission by GABA have been satisfied. For a compound to be considered as a neurotransmitter it should mimic the effect of the endogenous transmitter and be synthesised and localized in the approriate nerve terminals. A mechanism of inactivation should be present which treats the compound in the same way as the naturally released transmitter. Pharmacological agents should affect the compound and the neurotransmitter identically. Most difficult of all to study, the compound should be released in sufficient amounts to produce the physiological effect when the appropriate stimulus is applied. The aims of this study were:

1. To determine the ionic mechanisms of the responses of pyramidal neurones to the application of gamma-amino butyric acid (GABA) agonists.

2. To investigate the pharmacology of GABA with respect to hippocampal pyramidal neurones, including the action of the

benzodiazepines and GABA antagonists.

GABA was discovered in nature at the beginning of the century (Acherman and Kutscher, 1910) and was first detected in mammalian brain by Awapara, Landua, Fuerst and Seale (1950).

## 1.1. STATUS OF GABA AS A NEUROTRANSMITTER.

### 1.1.1. STATUS OF GABA AS A TRANSMITTER IN CRUSTACEANS.

Boistel and Fatt (1958) reported that GABA and the natural neurotransmitter both increased the conductance of the muscle fibre membrane to chloride ions in the claw opener muscle of the crayfish Astacus. When GABA was applied iontophoretically to muscle fibres of the abductor muscle of the dactylopodite of Cambarus walking leg, only the parts of the membrane underlying the inhibitory innervation were sensitive to the GABA (Takeuchi and Takeuchi, 1965). The effect of both GABA and the inhibitory neurotransmitter was to increase the chloride ion conductance of the membrane. Besides the muscle membrane, GABA and the inhibitory neurotransmitter increased the chloride ion conductance of the postsynaptic membrane in the stretch receptor organs of lobsters and crayfish (Hagiwara, Kusano and Saito, 1960).

Picrotoxin blocks the effect of inhibitory nerve stimulation and the effect of GABA on membrane conductance and muscle contraction, although at high (>10μM) concentrations (Van der Kloot, Robbins and Cooke, 1958). Picrotoxin is reported to antagonize GABA competitively (Grundfest, Reuben and Rickles, 1959), by a mixed competitive- non-competitive action (Constanti, 1978) but to be non-competitive on crayfish muscle (Takeuchi and Takeuchi, 1969). This action of

picrotoxin is reported to be less effective in media with low chloride ion concentrations (Takeuchi and Takeuchi, 1969). This evidence has been interpreted as an action of picrotoxin on the chloride channel in these preparations. GABA is synthesised in all organisms except viruses and Rickettsia by glutamic acid decarboxylase (GAD). In lobster muscles, GAD and GABA are concentrated in inhibitory nerve terminals (Hall, Bownds and Kravitz, 1970). There are uptake systems for GABA in crustaceans. Uptake is active and sodium ion dependent, but, unlike uptake in mammalian nervous systems (see below), GABA is taken up into Schwann cells but not into neurones (Iversen and Kravitz, 1968; Orkand and Kravitz, 1971). The experiments of Otsuka, Iversen, Hall and Kravitz (1966) bring the above points together as evidence of a transmitter role for GABA in crustaceans. They demonstrated that electrical stimulation of inhibitory nerves innervating muscles of the lobster caused a release of GABA proportional to the number of stimuli applied. The release was dependent on calcium ions. Stimulation of excitatory nerves had no effect on background release of GABA. There is therefore good evidence that GABA is a neurotransmitter in crustaceans, and that the mechanism of action is through an increase in conductance of the muscle fibre membrane to chloride ions.

### 1.1.2. STATUS OF GABA AS A NEUROTRANSMITTER IN MAMMALS.

As in crustaceans, GABA is synthesised by GAD. Both GABA

and GAD are concentrated in the CNS and the regional distribution of each are well correlated (Baxter, 1970). GAD may be concentrated in nerve terminals (Balazs, Hajos, Johnson, Reynierse, Tapia and Wilkin, 1975). GABA itself may play a role in CNS metabolism as the GABA - shunt (glutmate - GABA - succinate) accounts for about 10% of glucose metabolism (Patel, Balazs and Richter, 1970). Storm-Mathieson (1977) suggests that the high concentration of GABA in the brain is required solely to maintain neurotransmission, but Sytinsky, Soldatenkov and Lajtha (1979) believe GABA to have metabolic roles in the brain. Although GABA can be broken down (by GABA-transaminase), this is not thought to be the way in which GABA is inactivated in synapses; probably an uptake system is involved since there is a high affinity uptake system for GABA into brain slices (Bond, 1973) and also into cell free (synaptosomal) preparations. This uptake is sodium ion dependent (Iversen and Neal, 1968) and is unevenly distributed in the CNS, being found where GAD activity is high. Under conditions favouring the high affinity uptake, GABA-ergic neurones, including their axons and nerve terminals, are labelled with $^{3}$H-GABA (Hokfelt and Ljungdahl, 1972). However, glial cells may show uptake of GABA too and the best documented GABA neurones in the brain, the cerebellar Purkinje neurones, do not possess the uptake mechanism (Hokfelt and Ljungdahl, 1972).

In cell culture, spinal, brainstem and dorsal root

ganglion neurones respond to GABA with a hyperpolarization and a depolarization which are both inhibitory (Barker and Ransom, 1978). Dorsal root ganglion neurones in vivo are depolarized by GABA (De Groat, Lalley and Saum, 1972). Neurones in the cerebellar nuclei are hyperpolarized and inhibited (as shown by the decrease in the size of excitatory synaptic potentials) by GABA (Obata, Ito, Ochi and Sato, 1967), while neurones in the olfactory lobe are depolarized by GABA (Nicoll, 1971; Brown and Scholfield, 1979). Olfactory lobe neurones are inhibited (as shown by a decrease in the firing rate of the neurones) by GABA in in vivo experiments (Legge, Randic and Straughan, 1966). In this region, IPSPs are depolarizing (Scholfield, 1978) and it is possible that a reduction of the firing rate of these neurones in vivo is due to the depolarizing action of GABA. IPSPs recorded in the cerebral cortex have the same reversal potential as hyperpolarizing responses to GABA (Krnjevic and Schwartz, 1967) and so it appears that, in contrast to the olfactory bulb, the inhibitory effect of GABA may be due to a hyperpolarization of the neurone. Hippocampal neurones show biphasic responses to GABA which are inhibitory, although the IPSPs in these neurones hyperpolarize the neurone and it is likely that the physiological effect of GABA in the hippocampus is to hyperpolarize the neurone. It is interesting that GABA may inhibit neurones by both depolarizations and hyperpolarizations. The large conductance changes that accompany the responses to GABA, however, may shunt the effects of excitatory potentials or the currents underlying the action

potential and inhibit the neurone in this manner.

Where such studies have been done, the responses to GABA appear to be chloride dependent, although many of the studies are superficial and do not rule out the contribution from other ions (eg Scholfield, 1978). In bullfrog primary afferent neurones, the GABA mediated depolarizations are apparently mediated entirely by chloride ions, as the reversal potential of the response follows changes in the chloride ion concentration with in a manner predicted from the Nernst equation for chloride ions (Nishi, Minota and Karczmar, 1974) although Barker and Nicoll (1973) argued that sodium ions were involved in the response. The semilogarithmic relationship that they report betwen the external sodium ion concentration and the size of the response does not rule out the involvement of another ion. Indeed, Nishi, Minota and Karczmar (1974) criticize the experiments of Barker and Nicoll (1973) on these grounds and suggest that the effect of sodium substitution was to affect the internal concentration of chloride ions.

In the olfactory lobe, IPSPs produced by stimulation of the lateral olfactory tract were depolarizing and chloride dependent (as were responses to GABA). The involvement of a cation was not excluded (Scholfield, 1978). In the spinal cord, IPSPs recorded in motoneurones were chloride and potassium ion dependent (Coombs, Eccles and Fatt, 1955). Measurements of extracellular ion concentrations have shown

that the concentration of potassium ions increases during the application of GABA to the cerebral cortex (Brinley, Kandel and Marshall, 1969), the spinal cord (Kudo and Fukuda, 1976) and the hippocampal slice (Gutnick and Segal, 1981). This suggests that the hyperpolarizing responses to GABA were a result of potassium ions leaving the neurone.

While there is little difficulty in explaining an electrochemical gradient for potassium ions, chloride ions are often thought to be passively distributed. Electrophysiologically, this would result in the reversal potential of a chloride dependent response being equal to the membrane potential. In spinal motoneurones, however, Lux (1971) has demonstrated that $E_{Cl}$ was more negative than the membrane potential due to an outwardly directed pump. This resulted in hyperpolarizing IPSPs. Llinas, Baker and Precht (1974) reported an outwardly directed chloride pump in cat trochlear neurones that was reduced by ammonium ions. If there are apparently chloride dependent depolarizing and hyperpolarizing responses to GABA, then it is clearly important to rule out the involvement of other ions in the responses as these ions could change the sign of the response; in theory, it would be quite possible for a depolarizing response to be mediated by a hyperpolarizing chloride gradient and a more powerful depolarizing sodium gradient.

The use of bicuculline and picrotoxin has aided the

suggestion that GABA is a neurotransmitter. Thus bicuculline reduces the inhibition of firing of lateral geniculate neurones that GABA produced, at the same time reducing recurrent inhibition of these neurones (Curtis and Tebecis, 1972). Bicuculline reduces the basket neurone inhibition of Purkinje neurones in the cerebellum and the inhibition of the cerebral cortex produced by local stimulation (Curtis, Duggan, Felix and Johnston, 1970). Bicuculline also reduces the IPSP recorded in neurones of the olfactory cortex (Alger, Jahr and Nicoll, 1981). Bicuculline reduces the depolarization of dorsal root ganglion neurones produced by GABA (De Groat et al 1972) and the depolarization of the cuneate nucleus produced by GABA (Simmonds, 1980). In addition, bicuculline blocked the reduction of the firing rate produced by GABA in cortical and pallidal neurones (Perkins and Stone, 1982) and in spinal neurones (Allan, Evans and Johnston, (1980). If bicuculline is a specific antagonist of GABA, then it appears that GABA is involved in the physiology of inhibition at the sites mentioned, since IPSPs, responses to GABA and reductions in the firing rate of neurones by GABA are all blocked by bicuculline.

Many studies make no mention of which stereoisomer of bicuculline was used (eg Curtis et al 1970; Simmonds, 1980; Alger and Nicoll, 1982; Perkins and Stone, 1982). In ligand binding studies, however, (+)bicuculline is more effective in reducing the binding of GABA to rat brain (Tallman,(1978), so there may be differences between stereoisomers. Several of the

papers by Curtis and his colleagues show what appears to be the same stereoisomer as is given in the 'Merck Index' (page 1223, edition 9) as the (+) isomer. This is the isomer that occurs naturally. It seems likely that (+)bicuculline is the form more commonly used.

Picrotoxin is less useful than bicuculline as its mechanism of action is not known (see section 1.1). In the mammalian CNS, picrotoxin is effective in reducing the inhibition of firing that GABA produces in the cerebral cortex (Gallagher, 1978; Curtis, Duggan, Felix, Johnston and McLennan, 1971) and in the spinal cord (Curtis, Duggan, Felix and Johnston, 1971). Picrotoxin also blocks presynaptic inhibition in the spinal cord (Eccles, Schmidt and Willis, 1963). In addition, picrotoxin blocks the depolarization of dorsal root ganglion neurones (De Groat et al, 1972) and the cuneate nucleus (Simmonds, 1980) produced by GABA. In this latter study, picrotoxin antagonised GABA non-competitively. Non-competitive antagonism seems to be the norm in vertabrates, although not in crustaceans. Thus in lamprey spinal cord (Homma and Rovainen, 1978), dorsal roots of isolated frog spinal cord (Constanti and Nistri, 1976) and isolated spinal ganglia of the cat (Gallagher, Higashi and Nishi, 1978) picrotoxin antagonises the effect of GABA in a non-competitive manner. In ligand binding studies, picrotoxin reduces the binding of GABA and the benzodiazepines in a non-competitive manner. This reduction has a requirement for chloride ions

(Karobath, Drexler and Supavilai, 1981). Although there is no direct evidence for the hypothesis, picrotoxin is thought to act at the chloride ionophore (Fujimoto and Okabayashi, 1981); it seems likely that in vertabrates, picrotoxin does not act on the GABA receptor.

Having made these general points, it is convenient to look further at the evidence for GABA neurotransmission in the cerebellar Purkinje neurone projection to the dorsal part of the lateral vestibular nucleus (Deiter's nucleus), since studies of this pathway provide the best evidence of neurotransmission by GABA in the mammalian CNS. Impulses in the Purkinje neurone axons cause the inhibition of neurones in Deiter's nucleus (Ito and Yoshida, 1966). The same neurones are inhibited by a hyperpolarization and an increase in conductance when GABA is applied by iontophoresis (Obata, Ito, Ochi and Sato, 1967). GABA is found in high concentrations in Purkinje neurones and in the large neurones of Deiter's nucleus (Obata, Otsuka and Tanaka, 1970). GABA is released into the fourth ventricle when Purkinje fibres are activated (Obata and Takeda, 1969).

Picrotoxin and bicuculline act identically on the neurotransmitter and applied GABA; when applied iontophoretically (but not parenterally), picrotoxin blocks the Purkinje neurone inhibition of occulomotor neurones (Obata and Highstein, 1970).

Bicuculline reduces the effect of GABA on spinal, cerebral and cerebellar neurones, where GABA may be a transmitter (Curtis, Duggan, Felix and Johnston, 1970). Bicuculline applied by iontophoresis blocks the inhibition of Deiter's nucleus produced by stimulation of Purkinje neurones (Curtis, Duggan and Felix, 1970). The intravenous dose of bicuculline required to block synaptic inhibition in the Purkinje neurone pathway is about ten times the dose required to block postsynaptic inhibition in the spinal cord (Eccles, Schmidt and Willis, 1963).

GABA satisfies the criteria for a neurotransmitter and it is likely that the Purkinje neurone - Deiter's nucleus pathway utilises GABA as its neurotranmitter.

## 1.2. GABA AGONISTS AND THE BENZODIAZEPINES.

Many analogues of GABA have been synthesised in an attempt to separate different GABA receptors in the brain and spinal cord (Sytinsky, Soldatenkov and Ljatha, 1979). So far, there appear to be three GABA receptors in mammals. The $GABA_A$ receptor is activated by muscimol, blocked by bicuculline and is probably coupled to a chloride channel in the neuronal membrane. The $GABA_B$ receptor, activated by baclofen (β-chloro phenyl GABA) is not blocked by bicuculline and is found largely presynaptically (Bowery, Doble, Hill, Hudson, Shaw and Turnbull, 1980). The third receptor, found postsynaptically in the hippocampus by Alger and Nicoll (1982), is activated by THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol) and is blocked by high concentrations of bicuculline.

The widespread distribution of GABA sensitive (though not necessarily GABA innervated) neurones in the brain, spinal cord and periphery is both a blessing and a curse from the clinical point of view, although it provides the less clinical scientists with a range of useful preparations. The blessing is that the powerful inhibition of the CNS that GABA produces can be modified with drugs; so far, all have been used clinically to increase inhibition. The curse is that the lack of selectivity of GABA receptors in different regions of the CNS makes the targetting of a drug difficult. The $GABA_B$

receptor, however, has proved useful. Baclofen is a compound designed to activate this receptor and is used to control the spasticity produced by multiple sclerosis and some spinal injuries (Brogden, Speight and Avery, 1974).

No clinically useful drugs have been found that activate the $GABA_A$ receptor. GABA itself does not cross the blood-brain barrier. Although alkylation of the carbon atom of GABA improves brain penetration, the compounds produced are toxic. There is hope yet that agonists which activate the third GABA receptor type such as THIP (Alger and Nicoll, 1982) will prove useful in medicine.

The class of drugs most commonly prescribed in Britain, the benzodiazepines, interact indirectly with some, if not all, of $GABA_A$ receptors. The precise mechanism of the interaction is the subject of many symposia, but the evidence produced by ligand-binding experiments suggests that the benzodiazepine binds to part of the GABA receptor complex and causes a site to be unmasked that has a high affinity for GABA. The dependence of this process on chloride ions and the block of this process by picrotoxin suggest that a chloride channel is connected to the system. This three-part receptor model of GABA receptor, benzodiazepine receptor and chloride channel has been proposed by several groups (eg Tallman, 1980; Paul, Marangos and Skolnick, 1981) but as ligand binding studies do not necessarily represent the brain, electrophysiological studies

are necessary to correlate the potency of the benzodiazepines with their binding properties in in vitro preparations before this model of the action of benzodiazepines can be accepted as the clinical mechanism of action. The results of such electrophysiological assays are complicated. Most of the studies have used extracellular recording of firing rates and the effect of GABA on these firing rates as the basis for assaying the effects of the benzodiazepines. GABA reduces the firing rate of neurones. This inhibition is potentiated by the benzodiazepines (Nestoros, 1980; Nestoros and Nistri, 1979; Okamoto and Sakai, 1979; Steiner and Hummel, 1968). Some workers have found that the benzodiazepines depress the firing of neurones in the absence of exogenous GABA. Thus Steiner and Felix (1976) report that in the cat in vivo, diazepam injected locally reduced the firing rate of neurones in Deiters nucleus. Steiner and Felix (1976) found that diazepam reduced the inhibition of Deiter's nucleus produced by stimulation of Purkinje neurones in the cerebellum and also reduced the inhibition of the Purkinje neurones themselves which is produced by basket neurones in the cerebellum. Experiments involving intracellular recordings show both potentiation and block of responses of hippocampal pyramidal neurones to GABA (Biscoe, Duchen and Pascoe, 1983). In spinal cord neurones, benzodiazepines potentiated the depolarizing response to GABA (MacDonald and Barker, 1977). To enable the results of the various studies to be applied to man, behavioural tests in man should correlate with the binding properties in vitro. One

such study shows a correlation between the anxiolytic (a term coined for the benzodiazepines) activity in man of a range of benzodiazepines with the ability of the drugs to displace diazepam from human brain membranes (Braestrup, Albrechtsen and Squires, 1977).

Clinically, the usefulness of the benzodiazepines is limited by their side effects. The side effects are the result of widespread increases in inhibition in the CNS. Some newer benzodiazepines and their derivatives, the triazolopyridazines, may selectively activate subclasses of benzodiazepine receptor in parts of the brain. The search for such compounds was stimulated when two classes of benzodiazepine binding site were distinguished, one especially prevalent in the cerebellum and one in the rest of the brain (Supavilai and Karobath, 1980; Squires, Benson, Braestrup, Coupet, Klepner, Myers and Beer, 1979). Benzodiazepine binding site heterogeneity is suggested from the observation that the slopes of Hill plots were less than unity for displacement of [$^3$H]diazepam by certain non-benzodiazepine ligands (eg triazolopyridazines (Young, Niehoff, Kuhar, Beer and Lippa, 1981) and $\beta$-carbolines (Braestrup and Nielsen, 1981)). This data can also be interpreted as showing the presence of negative co-operativity, however. It is not known whether the different binding sites are different proteins or different forms of the same protein. A differential brain distribution, ontogenic developement, protein subunit molecular weight and suscepibility to the

action of detergents support the hypothesis of multiple binding sites (Braestrup and Nielsen, 1981; Young et al, 1981; Sieghart and Karobath, 1980; Regan, Roeske, Malick, Yamamura and Yamamura, 1981; Lo, Strittmater and Snyder, 1982). Heterogeneity is also apparent in binding kinetic studies, the sensitivity to affinity labels, protein reagents and heat inactivation (Olsen, 1982; Young et al, 1981; Sieghart and Karobath, 1980; Regan et al, 1981; Lo et al, 1982; Chiu and Rosenberg, 1983; Hirsch, Kochman and Sumner, 1982). Binding sites can also be separated according to their interaction with bicuculline and picrotoxin (Leeb-Lundberg and Snowman, 1981; Leeb-Lundberg and Olsen, 1982). Benzodiazepine binding sites may not be the receptors which mediate the therapeutic effects of the benzodiazepines, although, as mentioned above, Braestrup et al (1977) have shown that there is a good correlation between binding potency of the benzodiazepines to membrane preparations and their effects in man. In addition, the selectivity of the triazolopyridazines between binding sites is matched by their ability to produce the anxiolytic effects of the benzodiazepines without the ataxic and hypnotic side effects (Squires et al, 1979). Although the evidence is incomplete, it is likely that the therapeutic actions of the benzodiazepines are mediated by different receptors.

The complicated way in which the benzodiazepines act seems unlikely to be a mere evolutionary freak. There is thus a search for an endogenous ligand for the benzodiazepine

receptors. Various compounds are proposed: Inosine and hypoxanthine are proposed by Asano and Spector (1979) and β-carbolines are proposed by Braestrup, Nielsen and Olsen (1980). Although an effect of all these compounds on the binding of GABA to its receptor can be demonstrated, the β-carbolines differ from hypoxanthine and inosine in that they decrease the affinity of the GABA receptor for GABA, and act at concentrations in the nanomolar range (Nielsen and Braestrup, 1980). The reduction of the affinity of GABA receptors for GABA that is produced by the β-carbolines is of course opposite to the effect of the benzodiazepines. Various β-carbolines have been found in the brain. If the β-carbolines really are active in the brain, then the function of the GABA receptor complex may be to reduce inhibition rather than to increase it as medicine tries to do.

One of the subsidiary aims of the present study was to correlate the effect of the benzodiazepines in ligand-binding studies (from published data) with the potency of the drugs in potentiating a particular GABA response of one class of neurones. Another subsidiary aim was to investigate whether some of the responses to GABA were not chloride ion dependent and if these non-chloride dependent responses were potentiated by the benzodiazepines. If this were to be the case, then the involvement of a chloride channel in the GABA receptor complex with the benzodiazepine receptor would be in doubt. It was hoped that the variable action of picrotoxin (as mentioned

above) could be explained as a variable involvement of chloride channels in the responses.

Ethylenediamine (EDA) has been reported to have qualitatively similar effects to GABA when applied iontophoretically onto cortical neurones (Perkins and Stone, 1982). This may be due partly to an indirect action of EDA, as EDA can release GABA from brain slices and block the uptake of GABA (Forster, Lloyd, Morgan, Parker and Perkin, 1981; Lloyd, Perkins and Stone, 1982).

There is, however, evidence that EDA has a direct GABA-mimetic action. Thus bicuculline blocks the inhibition of rat pallidal neurones produced by the iontophoresis of EDA more effectively than it blocks the inhibition produced by GABA (Perkins, Bowery, Hill and Stone, 1981). EDA was less potent than GABA in this study. Perkins and Stone (1982) report in a more detailed study that the terminal carboxyl group of GABA is not required for action at the EDA receptor. They suggest that EDA is either a new type of GABA-mimetic (ie, that it acts on GABA receptors) or that that it acts on a new bicuculline-sensitive receptor.

In iontophoresis, EDA and GABA have similar transport numbers (about 0.15), allowing the potentcy of EDA to be roughly compared to the potency of GABA. When iontophoresed onto cortical neurones, EDA and GABA were about equipotent in

producing inhibition (Perkins and Stone, 1982). At high concentrations (1-10mM), EDA potentiates the binding of diazepam to cerebral cortical membranes in vitro (Morgan, Perkins and Stone, 1982). This evidence suggests that EDA may act on a site related to the normal GABA receptor, or that EDA may act on the GABA receptor itself.

Ethylenediamine was chosen as a GABA 'agonist' in this study for several reasons. First, GABA agonists normally require a carboxyl group for activity, and EDA has no such group. Secondly, EDA is more easy to obtain than muscimol or THIP. Thirdly, Dr. T. W. Stone suggested that the effects of EDA on hippocampal neurones might be interesting. This study compares the data obtained with GABA with the data obtained with EDA where possible, in an attempt to assess the value of EDA as a GABA agonist.

## 1.3. THE HIPPOCAMPUS AND THE STATUS OF GABA AS A NEUROTRANSMITTER IN THE HIPPOCAMPUS.

### 1.3.1. ANATOMY.

The hippocampus has the shape of a long cone and, in rats, is curled inside the cerebral cortex. The hippocampus arises in the dorsal midline and curls dorsally before descending towards the amygdala. It is easy to separate the hippocampus from the rest of the brain during a dissection because the alveus (the white matter that carries efferent and afferent fibres) forms part of the wall of the fourth ventricle. When removed and stretched flat, the hippocampus has the appearance of a long cone. If transverse sections of the hippocampus are made, then many of the integral, afferent and efferent pathways remain intact as the hippocampus has a lamellar organisation (Andersen, Bliss and Skrede, 1971). Examination of the cut surface reveals two distinct neurone layers. These layers are the pyramidal neurone layer and the granule neurone layer. Figure 1.1A shows this arrangement. The dendrites and axons of the pyramidal neurones extend at right angles to the pyramidal neurone layer and remain in one slice. The dendrites projecting towards the alveus are the basal dendrites, and the axons of the pyramidal neurones branch from these. Some of the axons leave the hippocampus by the subiculum and some by the fimbria. It is possible to distinguish five layers parallel to the pyramidal neurone layer. These five layers, for CA1 neurones (Cornu Ammonis region 1, after the nomenclature of

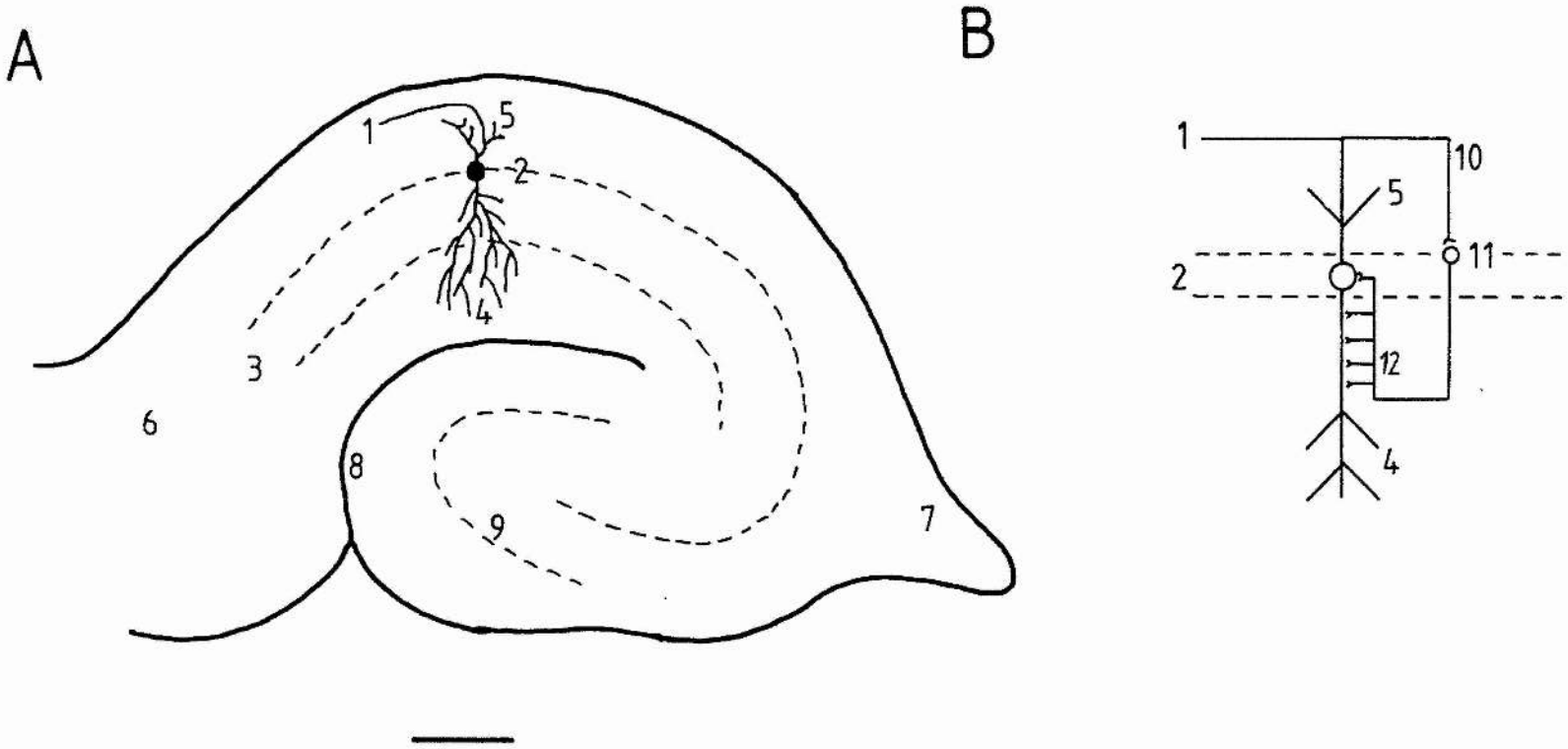
FIGURE 1.1.

A. Diagramatic view of a hippocampal slice.

Scale bar 1cm.

B. Schematic enlargement of 1 pyramidal neurone and 1 basket cell.

1. Pyramidal neurone axon.
2. Pyramidal neurone layer.
3. Stratum radiatum dendrites.
4. Apical dendrites.
5. Basal dendrites.
6. Entorhinal area.
7. Fimbria.
8. Fissure.
9. Granule neurone layer.
10. Axon colateral.
11. Basket cell.
12. Basket cell axon and nerve terminals.


A
1
5
2
4
3
6
8
9
7
B
1
10
5
2
11
12
4

Lorente de No, 1934), are the alveus, the stratum oriens, the stratum pyramidale (cell body layer), the stratum radiatum and the stratum moleculare-lacunosum.

The pyramidal neurone axons are the only efferents of the hippocampus and project to the septum, thalamus, entorhinal cortex and subiculum. The CA1 pyramidal neurones receive inputs from:

1. the contralateral hippocampus, via the fimbria, to the stratum oriens and stratum radiatum,
2. the ipsilateral CA3 and CA4 pyramidal neurones (the Schaffer collaterals) to the stratum radiatum,
3. basal levels (probably the prepyriform cortex) to the stratum moleculare,
4. the locus coeruleus and Raphe nuclei to the stratum radiatum,
5. amygdaloid nuclei to the stratum molecular.

In addition to the pyramidal neurones and granule neurones, the hippocampus contains interneurones. Basket neurone interneurones are found apical to and basal to the pyramidal neurone layer. Basket neurone axons descend into the apical dendrites of the pyramidal neurones and then turn back, branching profusely, and synapse with the apical dendrites and cell body of the pyramidal neurones. Interaction between the hippocampal lamellae takes place through these interneurones. Figure 1.1B shows the arrangement of the basket neurones.

## 1.3.2. ELECTROPHYSIOLOGY.

The granule and pyramidal neurones are excitatory and receive excitatory inputs from the entorhinal area, the ipsi- and contra-lateral hippocampus and the septum. The septal input may pace the hippocampal theta rhythm which is observed in intact animals. Studies of the field potentials generated by these inputs suggest that excitatory post-synaptic potentials (EPSPs) are produced in the dendrites.

Hippocampal neurones can be inhibited by stimulation of the locus coeruleus, the Raphe nuclei and the cerebellar nucleus fastigii (Heath and Harper, 1974). A powerful recurrent inhibition of pyramidal neurones is produced by the basket neurones when they are stimulated by pyramidal neurone axon collaterals (Andersen, Eccles and Loyning, 1964).

Assaf, Crunelli and Kelly (1981b) report that small amplitude spikes are found in granule neurones in the hippocampus; local dendritic action potentials were thought to correspond to the small spikes recorded in the soma. Other small spikes have been reported in hippocampal pyramidal neurones, and were thought to be products of a spike initiation zone (Kandel, Spencer and Brinley, 1961). In addition to these, fast pre-potentials which precede the small spikes were seen. The conclusion reached was that these fast pre-potentials were produced by an active membrane in the

dendrites, and that a passive membrane lay between this region and the cell body. Purpura, McMurty and Malliani (1966) and Andersen and Lomo (1966) report a dendritic origin of small spikes in pyramidal neurones. Schwartzkroin (1975) also reports dendritic spikes in pyramidal neurones. The terminology is confusing, but most authors give some reference as to the origin of the potentials, calling them D-spikes, dendritic spikes.

In extracellular studies of hippocampal neurones, spike-like potentials have been seen in response to afferent stimulation (Andersen, 1960; Cragg and Hamlyn, 1955) which were ascribed to activity in excitable dendrites. Eccles (1957) has suggested that the main apical dendrite branch would be a good location for dendritic spikes to be produced. In hippocampal neurones, this bifurcation is between 100 and 700μm from the cell body (Lorente de No, 1934). Motoneurones are different from pyramidal neurones in that their dendrites are passive, without regions of excitability and with a longer length constant (Barrett and Crill, 1974).

If there are spikes in the dendrites of the pyramidal neurones which are decrementally conducted to the cell body, then an examination of the electrotonic parameters of the neurones might suggest whether such decremental conduction could occur. According to the modelling of Rall (1959), theoretical models of CA1 hippocampal pyramidal neurones have

been made by Traub and Llinas (1979), Turner and Schwartzkroin (1980), and Brown, Fricke and Perkel (1981) and of CA3 pyramidal neurones by Johnston (1981) and Brown, Fricke and Perkel (1981). All the studies of CA1 neurones conclude that the neurones are electrically compact, although the models produce slightly different results. Of particular interest to the study of dendritic spikes is the electrotonic length (L) of the dendrites. Traub and Llinas (1979) report L = 0.8, Turner and Schwartzkroin (1980) report L = 1.5 and Brown, Fricke and Perkel (1981) report L = 1.0. Given the problems and the assumptions of the models, these measurements can be taken as roughly the same (Brown, Fricke and Perkel, 1981). These figures give about a 1% transfer of voltage from distal dendrites and only a few % from more proximal sites (Brown, Fricke and Perkel, 1981; Turner and Schwartzkroin, 1980). Brown, Fricke and Perkel (1981) also conclude that the dendrites are excitable. Their conclusions are supported by Wong and Prince (1979) who impaled the initial segments of the dendrites of hippocampal pyramidal neurones and reported the presence of action potentials. It is not inconceivable that action potentials in the dendrites of pyramidal neurones even if they were 50mV in size, would be decrementally conducted to the cell body recording site and seen as only a few mV in size.

### 1.3.3. NEUROTRANSMISSION BY GABA IN THE CA1 REGION.

The enzyme GAD is found in most layers of the CA1 region, but is concentrated round pyramidal neurone cell bodies and in

the stratum radiatum. The distribution coincides with the distribution of the cell bodies and synapses of basket neurones. GABA uptake, measured autoradiographically, occurs around (but not into) pyramidal neurones. Heavily labelled neurones are scattered throughout all the layers (Iversen and Schon, 1973). Endogenous GABA is found with the same distribution as GAD. GABA (and glutamate) have recently been visualized immunocytochemically (Storm-Mathieson, Leknes, Bore, Vaaland, Edminson, Haug and Ottersen, 1983). The distribution of GABA by this method is the same as that reported by Iversen and Schon (1973).

The levels of GAD and GABA are changed little by lesions of hippocampal afferents or efferents (see Storm-Mathieson 1977 for a review). This suggests that the levels of GAD and GABA are dependent on intrinsic neurones.

It appears likely that basket neurones use GABA as their transmitter but direct evidence is lacking.

Pyramidal neurones are inhibited by the application of GABA and both this inhibition and the inhibitory post-synaptic potential (IPSP) produced by antidromic activation are blocked by bicuculline (Curtis, Felix and McLennan, 1970; Alger and Nicoll, 1982). The IPSP is inverted by the intracellular injection of chloride ions (Alger et al, 1981) and the careful study by Allen, et al (1977) suggests that the IPSP is chloride

dependent.

The GABA receptors on hippocampal pyramidal neurones have a high affinity for analogues of GABA that contain the extended form of GABA (Segal, Sims and Smissman, 1975), which suggests that the receptors are of the $GABA_A$ type.

From intracellular studies, several groups have reported that the responses of hippocampal pyramidal neurones to GABA are complex (Langmoen, Andersen, Gjerstad, Laursen and Ganes, 1978; Alger and Nicoll, 1979; Blaxter and Cottrell, 1982).

The mapping experiments of Andersen, Dingledine, Gjerstad, Langmoen and Laursen (1980) show that the dendrites of the stratum radiatum region respond with a depolarization to the iontophoresis of GABA and that the cell body responds with a hyperpolarization. The hyperpolarization has a similar reversal potential to the IPSP (Alger and Nicoll, 1982; Andersen et al, 1980).

Alger and Nicoll (1982) extended the observations of Andersen et al (1980) pharmacologically. The depolarizing response to GABA is reduced by bicuculline (1μM) and the hyperpolarizing response is reduced by a high concentration (300μM) of bicuculline. Diazepam potentiates both responses, the hyperpolarization being more susceptible. In addition to the usual depolarizing response of the dendrites to GABA, there

was an occasional hyperpolarization of the dendrites in response to GABA. This hyperpolarization was often found when THIP was applied.

The status of THIP as a GABA agonist is not clear, since it does not stimulate the binding of benzodiazepines to neuronal membranes as GABA, muscimol and a range of other GABA agonists do (Maurer, 1979). It is thought, however, that THIP activates subsynaptic receptors rather than the common extrasynaptic receptors (Allan, Evans and Johnston, 1980).

Despite the various responses to GABA in the hippocampus, Bormann, Sakmann and Seifert (1983) report only one population of GABA-activated channels in the cell body membrane of rat hippocampal neurones in culture, the channels being chloride channnels.

Tsuchiya and Fukushima (1978) reported that the recurrent inhibition of hippocampal neurones brought about by antidromic stimulation in vivo is potentiated by diazepam given parenterally. The effects of flurazepam in the hippocampal slice preparation are, however, less clear; some responses are reduced and some are potentiated by flurazepam (Biscoe, Duchen and Pascoe, 1983). Jahnsen and Mosfelt Larsen (1981) report that responses of hippocampal pyramidal neurones were potentiated by benzodiazepines, although they do not indicate which, if any, of the responses were most susceptible.

The hippocampal slice was used in the present study for several reasons. First, the expertise was already present in the laboratory. Secondly, the hippocampal slice is easy to work with because the neurones in the slices are neatly arranged. Thirdly, GABA has been fairly well established as a neurotransmitter in the hippocampus. Fourthly, there seemed a good chance of testing the effects of benzodiazepines on a GABA response that was not chloride dependent and lastly, as the main interest was in GABA, the extensive literature on GABA in the hippocampus was considered helpful.

# CHAPTER TWO

# METHODS

The descriptions of the Methods are arranged in the following order: The permanent fixtures of the laboratory, the electronic apparatus, the bath and recording chamber, the preparation of the brain slices and finally their use and the way in which the experimental variables were manipulated.

## 2.1 FIXTURES.

Impalements of pyramidal neurones were made on a vibration-free bench. Four benches were used with varying success, which was dependent on their position in the building. All were designed to support a concrete slab (100kg) which was placed on top of the bench and supported by steel angle iron. Composite plastic foam (from instrument packaging) was placed between the slab and the steel. The initial thickness of the foam of 5cm was compressed to about 1cm by the weight of the slab. A steel baseplate (50kg) was positioned on the slab and rested on more of the foam. The thickness of the foam under the baseplate was critical; the more there was, the less vibration was detectable. The amount was limited by the tendency of the baseplate to swing. The massive parts of the bench limited the transmission of low frequency vibration and the foam limited the transmission of high frequencies. Foam proved to be more effective than bicycle inner tubes, rubber pressure hose or "Neurides"; a commercial system that was basically a number of small (20cm) diameter inner tubes. Each arrangement was tested initially with an accelerometer and this proved to be a useful indication of the ease with which impalements could be maintained when experiments were started.

## 2.2 ELECTRONIC APPARATUS.

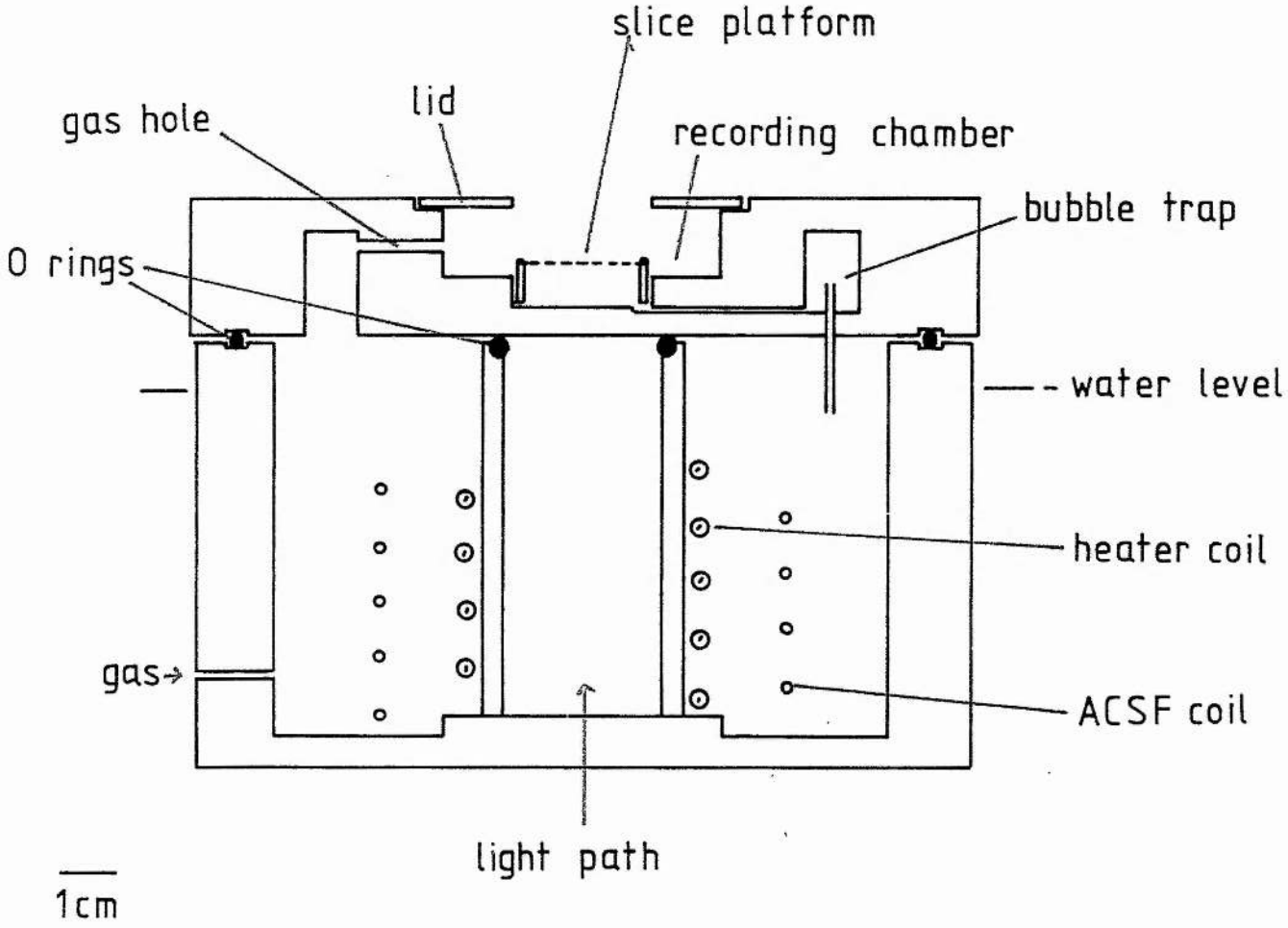
Intracellular potentials were amplified *via* the standard headstage by a Neurolog referenced DC pre-amplifier with a bridge circuit (NL102) or a similar ground referenced pre-amplifier (NL102G). The signal from the 10x output was displayed on a Tektronix 502A oscilloscope. The stimulator was a two channel Grass S88. One channel was used to pass pulses to the bridge (usually 0.02V to 0.15V for 100ms at 1 Hz) to give constant current hyperpolarizing pulses across the neuronal membrane; the second channel either switched the iontophoresis programmer (WPI model 160) on and off or provided stimuli to the preparation *via* a monopolar tungsten wire electrode. Membrane potential was recorded when required on one channel of a Gould 220 chart recorder. The signal was taken from the amplified (10x) output of the oscilloscope. The other channel of the chart recorder displayed the stimulator monitor (directly from the stimulator).

## 2.3 RECORDING CHAMBER.

Initially a large machined Perspex bath was used. It was a modification of a design by Richards and Sercombe (1970). As FIG 2.1 shows, the bath was a cylinder about 15cm in diameter. Inside this was a concentric cylinder which provided support for the heating coil. This coil ( 1m of Nichrome wire, 12Ω/m) inside 2mm silicone tubing) was supplied with a DC source (6V, 1A) from a constant current power supply (Farnell). The power was switched on and off by a temperature controller which measured the temperature of the water bath surrounding the heating coil with a thermistor (Radiospares GL23 2kΩ). The temperature was maintained at $33^{\circ}C \pm 1^{\circ}C$ The lid of the bath was machined so that a well 6cm across protruded into the airspace above the water level in the water jacket. The well was drilled in eight places to allow the gas (95% $O_2$, 5% $CO_2$) to bubble through the water jacket into the bath and over the slices. The slices were placed on a circular mesh in the centre of the well. The gas, which was warmed and wetted by the water jacket, supplied oxygen and carbon dioxide to the top surface of the slice and prevented it from drying out. The slice was perfused from below with artificial cerebro-spinal fluid (ACSF). The ACSF had only one path to waste - up round the slice, over the edge of the net and into the suction system. The nets were pieces of nylon tights, stretched across the rim of a cylinder of Perspex (2.5cm diameter, 0.5cm high) and glued in place with Araldite epoxy adhesive. The bottom of

FIGURE 2.1.

Vertical section through the circular bath and recording chamber.


slice platform
lid
gas hole
recording chamber
bubble trap
O rings
water level
heater coil
gas
ACSF coil
light path
1cm

the slice platform was a tight push fit into a depression machined into the bottom of the well and it was thus removable, enabling the slices to be attached to the the slice platform and stored outside the recording chamber. With a slice in position, the well was covered with a lid which allowed the light and the microelectrode to reach the slice. The lid reduced gas and heat loss and minimised air currents around the microelectrode. The ACSF entered the bottom of the water jacket inside a polythene tube (Portex O.D. 1.25mm). This tube spiralled inside the water jacket and entered the recording chamber under the slice platform. The length of the spiral in the jacket was 50cm and, as at the usual perfusion rate of 1 - 3 ml/min the ACSF spent 30s in the spiral, there was enough time for the ACSF to warm up. The dead volume of the spiral and the ACSF under the slice platform was 3ml. ACSF was changed under the slice in about 7 min at a perfusion rate of 3ml/min. This was measured as the clearance time of a dilute solution of methylene blue.

A Ag/AgCl wire cemented to the bottom of the chamber provided the indifferent electrode.

There were three problems associated with this bath:

1. The time spent in waiting for solutions to change represented too large a proportion of the total impalement time and limited the number of changes of ACSF that could be made to one neurone.

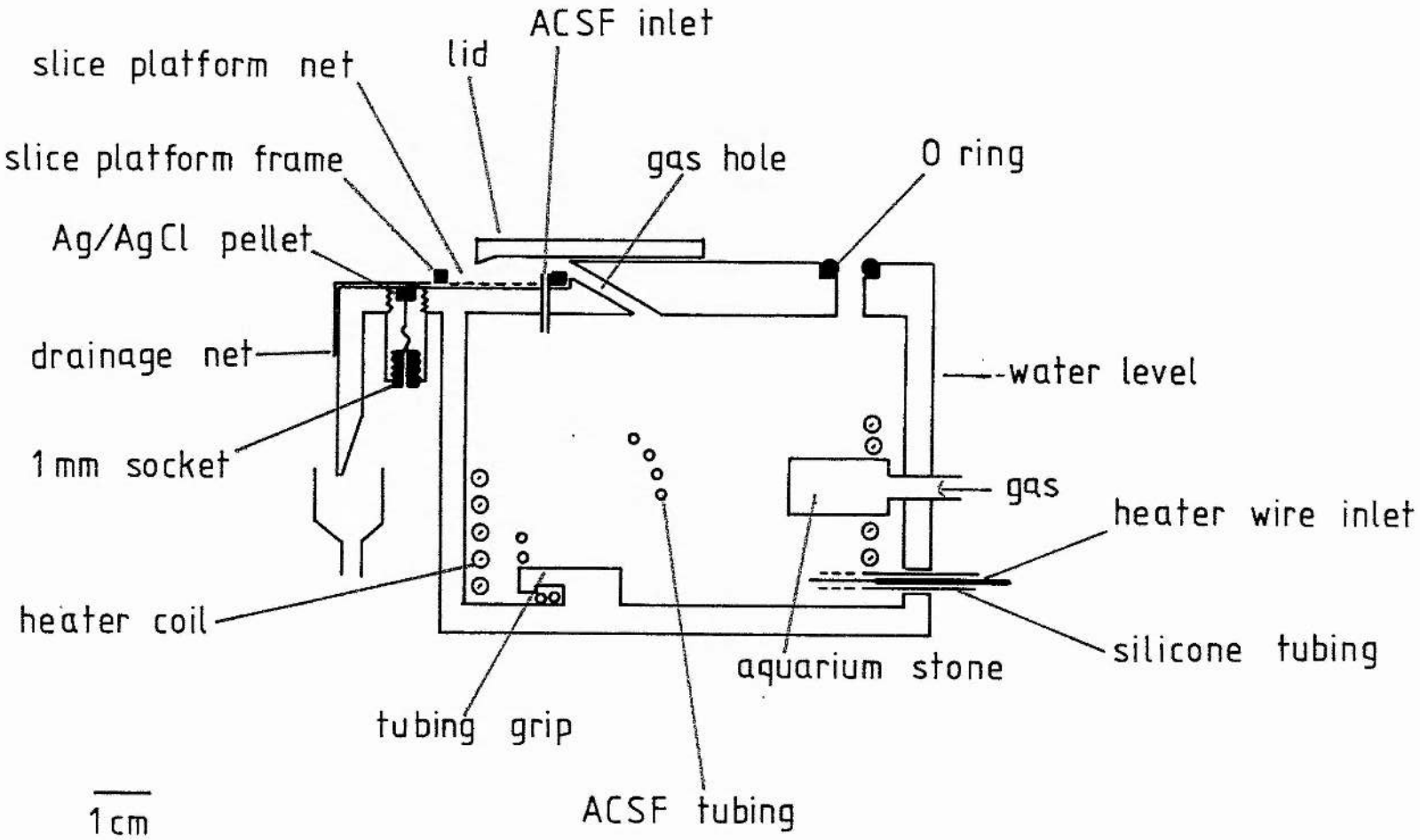
2. Impalements were often short (10 - 30 min) because moving the tap, despite its excellent design (Cobbett, 1981), interrupted the flow of ACSF long enough for the net supporting the slice to subside.

3. Cleaning the water jacket and recording chamber were time-consuming tasks as they involved extensive washing and rinsing *in situ*.

The need to maintain impalements reliably for 1h or more and to use impalement time as efficiently as possible became apparent on attempting quantitative experiments with the benzodiazepines. For these reasons, a very simple bath described by Haas, Schaerer and Vosmansky (1979) was tried as a substitute for the machined, circular bath. The Haas bath (FIG 2.2), as it is commonly known, was made from sawn sheets of Perspex and required no machining. Machined Perspex is susceptible to stress cracks. In principle it was the same as the circular bath, with a water jacket lying under the recording chamber. The gas mixture was bubbled through this jacket and the perfusion lines spiralled through the warm water. The perfusion rate was the same at 1 - 3ml/min. The Haas bath was different in that the dead volume was 200µl (this being the tubing volume) as the net that supported the slice sat on the bottom of the recording chamber. The ACSF entered the recording chamber through a stainless steel tube which stood proud of the bottom of the chamber to prevent bubbles from being trapped under the slices. The ACSF flowed by

FIGURE 2.2.

Vertical section through the Haas bath and recording chamber.


ACSF inlet
lid
slice platform net
slice platform frame
gas hole
O ring
Ag/AgCl pellet
drainage net
water level
1mm socket
gas
heater wire inlet
heater coil
silicone tubing
aquarium stone
tubing grip
ACSF tubing
1cm

capillary action through the narrow gap between the slice and the recording chamber bottom. Most of the instability due to changes in the perfusion rate were eliminated as the slices were not supported on a liquid surface. The slice platform for the Haas bath was rectangular. The frame was made from strips of Perspex and nylon net was glued to the underside of the frame with Araldite epoxy adhesive. The bath could be dismantled easily for cleaning and repair.

## 2.4 ARRANGEMENT OF THE BASEPLATE.

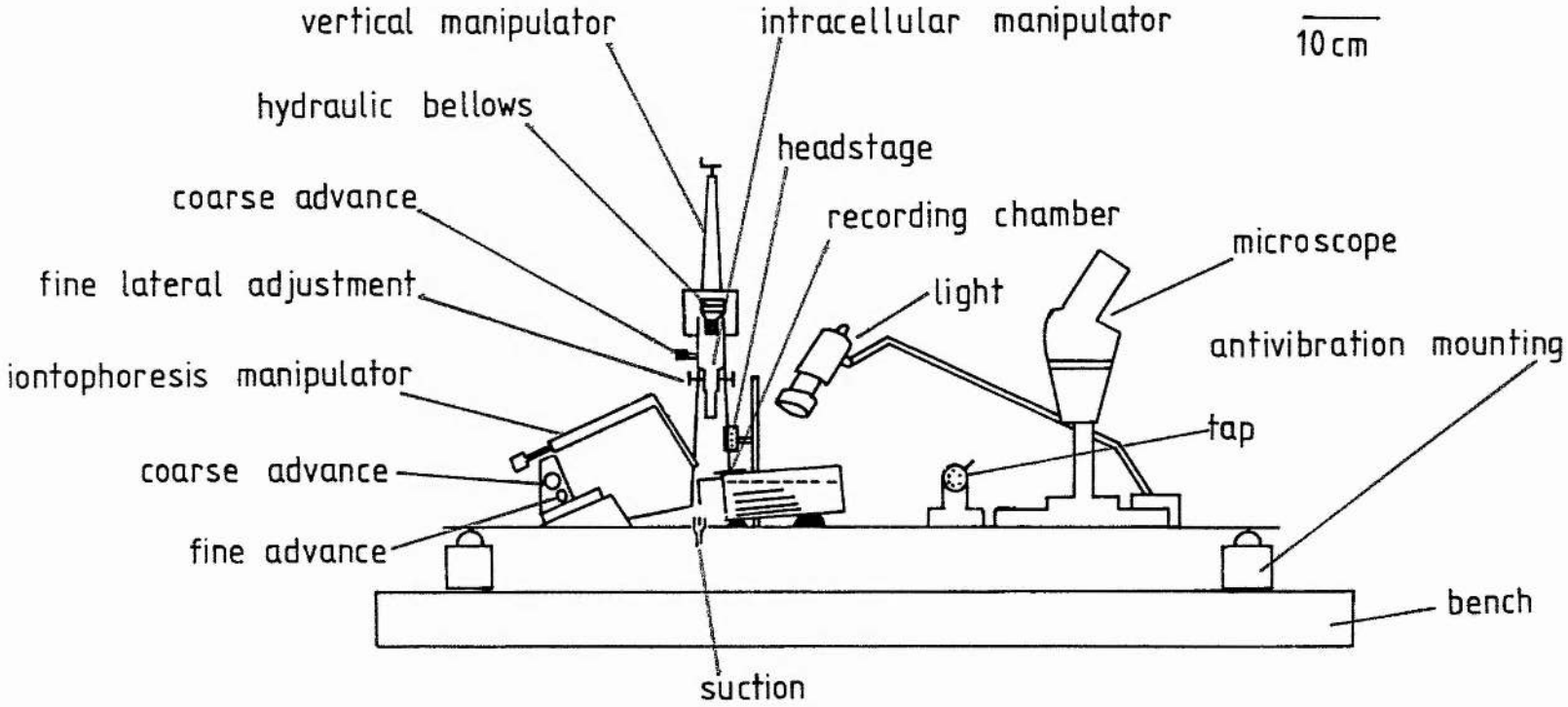
The arrangement is shown in FIG 2.3. The intracellular electrode manipulator was hydraulically advanced by a lever remote from the baseplate. The electrode could be advanced coarsely in the axis of the electrode by a worm gear arrangement. The manipulator was mounted on a heavy stand.

A Prior manipulator was used for the iontophoresis electrode. The manipulator moved in y (coarse and fine), x and z planes relative to the axis of the electrode. The angle of incidence relative to the slice of both intracellular and iontophoretic electrodes was made to be the same since this simplified the positioning of the electrodes relative to each other.

The microscope (Nikon) was a stereo zoom (10x to 40x) with a long working distance objective. Its heavy base allowed it to be swung out for access to the bath. To aid drainage of the recording chamber, the Haas bath was set in Plasticine on the baseplate at a slight slope. A suction system was connected to the bath through the baseplate to reduce the aerial effect of the saline-filled tube. The bath electrode was connected to earth by a flexible lead that plugged into the underside of the recording chamber. The ACSF flowed through the 3-way tap into a short section of silicone tubing, where a tubing clamp regulated the perfusion rate.

FIGURE 2.3.

Stylized elevation of baseplate arrangement from the front.


vertical manipulator
intracellular manipulator
10 cm
hydraulic bellows
headstage
coarse advance
recording chamber
microscope
fine lateral adjustment
light
antivibration mounting
iontophoresis manipulator
tap
coarse advance
fine advance
bench
suction

## 2.5 ARTIFICIAL CEREBRO-SPINAL FLUID.

Various salines have been used in the past to perfuse brain slices (see TABLE 1). Those used by Schwartzkroin (1975) and Alger and Nicoll (1982) have a higher $[K^+]$ than cerebro-spinal fluid (Ames, Sakenove and Endo (1964). The use of a more natural $[K^+]$ reduces the synaptic noise and the rate of firing and produces more reliable intracellular recordings (Langmoen and Andersen, 1981). The ACSF was made up from stock solutions. The stock solutions were replaced every two weeks and stored at $4^oC$ in bottles which had been washed in 95% ethanol and dried. No effect of bacterial, algal or fungal infection was seen. Calcium salts precipitated out of old stock solutions. Glucose was added as a solid just before final dilution. The concentration of the ions in modified ACSFs is shown in TABLE 2.

To increase the concentration of an ion in the ACSF the appropriate salt was added. Reductions of the concentration of an ion was accomplished by substituting a non-permeant ion. Thus for low $Na^+$ ACSF, NaCl was substituted with choline chloride and for low $Cl^-$ ACSF, NaCl was substituted with sodium isethionate. On several occasions NaCl was substituted with twice the molarity of sucrose (to maintain the osmotic strength). For low $K^+$ ACSF, all the KCl was omitted from the ACSF, and only half the $KH_2PO_4$ was added.

TABLE 1

Concentration of ions in the ACSF used by various workers.

Concentrations in mM. CSF; Ames, Sakenove and Endo, 1964. Alger; Alger and Nicoll, 1982. Schwartzkroin; 1975. Langmoen; Langmoen and Andersen, 1981. Blaxter, present study.

| | CSF | Nicoll | Schwartzkroin | Langmoen | Blaxter |
|---|---|---|---|---|---|
| $Na^{+}$ | 158 | 144.4 | 150 | 153 | 150 |
| $K^{+}$ | 2.7 | 7.9 | 6.25 | 3.25 | 3.75 |
| $Ca^{++}$ | 1.5 | 2.5 | 2 | 2 | 2 |
| $Mg^{++}$ | 1.3 | 1.3 | 2 | 2 | 2 |
| $Cl^{-}$ | 144 | 126.7 | 133 | 133 | 130.5 |
| $HCO_3^{-}$ | | 26.2 | 26 | 26 | 26 |
| $SO_4^{--}$ | | 1.3 | 2 | 2 | 2 |
| $PO_4^{--}$ | | 0.9 | 1.25 | 1.25 | 1.25 |
| Glucose | | 11 | 10 | 10 | 10 |

TABLE 2

Composition of solutions used in the present experiments (mM).

| | normal | low $Na^+$ | low $Cl^-$ | low NaCl | low $K^+$ | low $Ca^{++}$/ high $Mg^{++}$ |
|---|---|---|---|---|---|---|
| $Na^+$ | 150 | 26 | 150 | 26 | 150 | 150 |
| $K^+$ | 3.75 | 3.75 | 3.75 | 3.75 | 0.63 | 3.75 |
| $Ca^{++}$ | 2 | 2 | 2 | 2 | 2 | 1 |
| $Mg^{++}$ | 2 | 2 | 2 | 2 | 2 | 10 |
| $Cl^-$ | 130.5 | 130.5 | 88 | 130.5 | 128.5 | 129.5 |
| | | | 72 | | | |
| | | | 6 | | | |
| $HCO_3^-$ | 26 | 26 | 26 | 26 | 26 | 26 |
| $SO_4^{--}$ | 2 | 2 | 2 | 2 | 2 | 10 |
| $PO_4^{--}$ | 1.25 | 1.25 | 1.25 | 1.25 | 0.63 | 1.25 |
| choline | - | 124 | - | - | - | - |
| isethionate | - | - | 42.5 | - | - | - |
| | | | 58.5 | | | |
| | | | 124.5 | | | |
| sucrose | - | - | - | 124 | - | - |
| glucose | 10 | 10 | 10 | 10 | 10 | 10 |

## 2.6 PREPARATION OF THE BRAIN SLICES.

### 2.6.1. THE VIBRATOME.

A Gillette "Platinum" razor blade was split in half and one piece clamped in the slicing head of an Oxford Vibratome. The head was aligned so that the blade pointed down by $10^{o}$. The cutting chamber was filled with ACSF until the blade was covered by about half a centimetre of saline. Gas (95% $O_2$, 5% $CO_2$) was introduced into the chamber by a rigid plastic tube which had small holes punched in it. The bubbles stirred and oxygenated the ACSF. Hot water (48 - 50 $^{o}C$) was circulated through the cutting chamber inside a glass coil. A pump supplied water from a 3 l reservoir.

### 2.6.2. THE DISSECTION OF THE BRAIN.

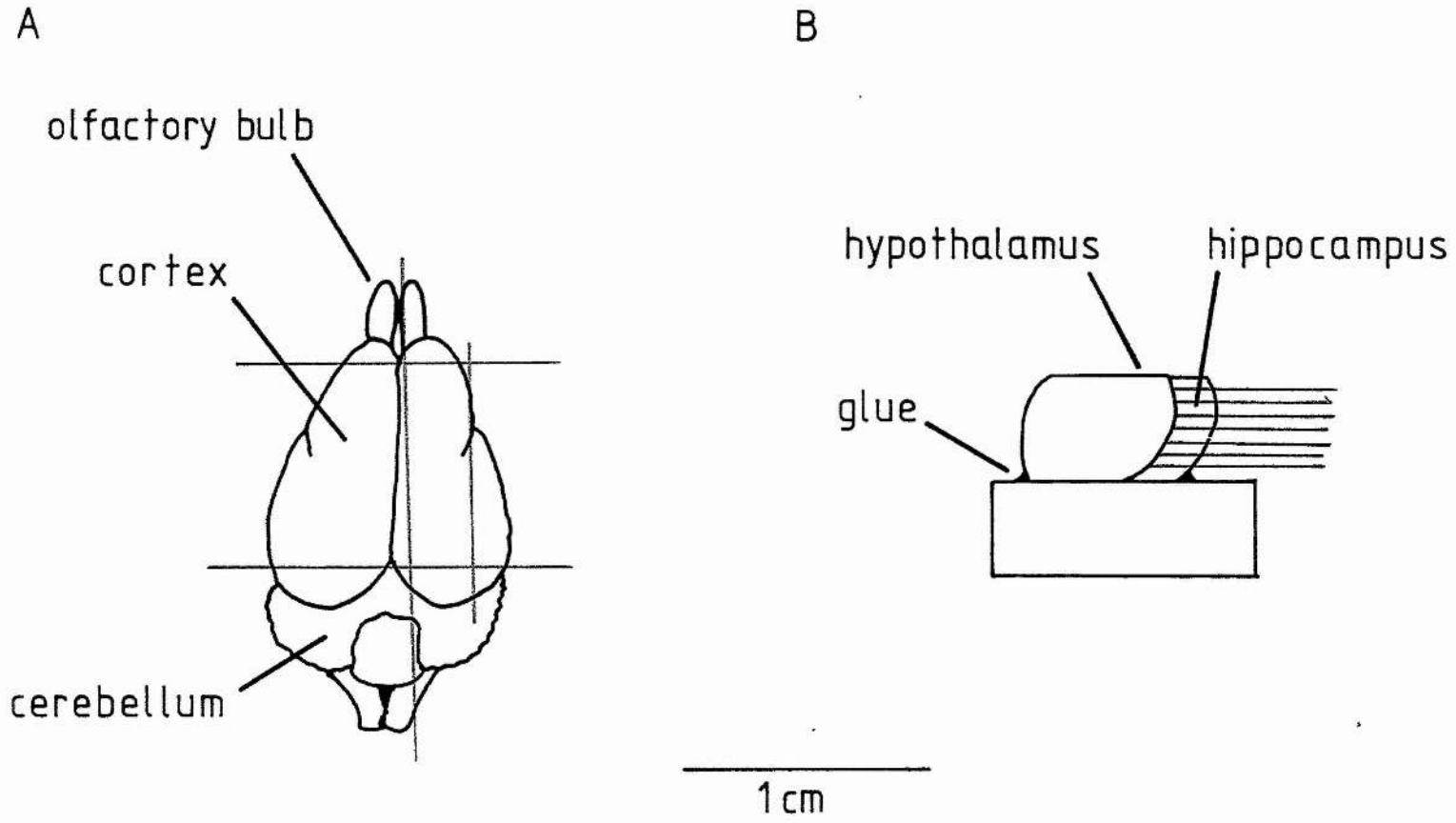
Male rats (150 - 250g) were killed either with a blow to the neck with an iron bar after stunning or by asphyxiation in $CO_2$. The rat was killed when the temperature of the ACSF in the cutting chamber and the temperature of the water in the reservoir the same at $37^{o}C$. The rat was placed on a cork board and the skin opened from the back of the neck to a point in front of the eyes. Two large pins held the skin away from the skull and anchored the head as the cranium was cut on the midline from the foramen magnum to the area over the olfactory lobes and both sides of the skull pulled away with serrated forceps, exposing the dura and underlying brain. The dura was

carefully cut away, leaving the brain sitting in the lower half of the skull. It was removed with a curved spatula. The olfactory bulbs and often the cerebellum were left behind. The brain was transferred to a beaker containing warm ACSF from the Vibratome cutting bath and washed free of surface blood. The brain was removed from the beaker to a damp filter paper. Enough ACSF was taken over with the brain to stop the tissue sticking to the paper. Four cuts were made in the brain to isolate the block of tissue containing the right hippocampus and surrounding structures (neocortex, hypothalamus, entorhinal cortex and fimbria). The placing of these cuts is shown in FIG 2.4A. This block was then lifted away with the razor blade and glued to a small aluminium block with cyanoacrylate adhesive (Radiospares). This adhesive is polymerized by the presence of water and so is ideal for wet tissue. It is also non - toxic. The lateral surface of the block was stuck down, leaving the medial face uppermost. A transverse section of the medial hippocampus could then be clearly seen. Using the corner of the razor blade, the neocortex and the heavy myelin of the corpus callosum was peeled away, exposing the dorsal part of the hippocampus with its characteristic silver-coloured alveus. While removing these tissues involved some risk of nicking the hippocampus, the cutting of the slices was more rapid as tough myelin did not have to be negotiated. The aluminium block and the trimmed tissue block were transferred to the Vibratome bath and clamped to a movable stage. Thus a brain at $37^{o}C$ was tranferred to the cutting chamber at about $36^{o}C$ and, during

FIGURE 2.4.

A. Dorsal view of rat brain. Red lines show position of cuts that isolate the right hippocampus.

B. Section through mounted block of brain showing how the angle of section of the slices changes as each slice is cut.


A
olfactory bulb
cortex
cerebellum
B
hypothalamus
hippocampus
glue
1 cm

cutting of the slices, cooled down with the rest of the system to about 33°C. This was the temperature of the storage bath and the temperature at which recordings were made. In this way thermal shocks to the brain slices were minimised.

2.6.3. CUTTING THE SLICES.

The blade was advanced as slowly as possible (about 1mm/min) and with the maximum amplitude of lateral vibration. The latter was achieved in excess of that of the original machine by mounting the block which held the razor blade on thick rubber washers. The lateral amplitude was about 2mm at 50Hz. The first slice was cut thick enough to leave a smooth, horizontal surface behind. This slice was discarded. The hippocampus starts near the midline of the brain and rises dorsally before curling round inside the cortex and ending ventrally near the amygdala. The top slice was usually thick enough to remove all the remaining hippocampus between the midline and the most dorsal part of the arch. This was necessary as the neuronal layers in this region were not clearly defined and impalements were consequently difficult. The slices became more oblique sections as the lateral hippocampus was cut (FIG 2.4B). This change in sectioning angle did not appear to interfere with the lamellar arrangement of the hippocampus, as single blood vessels could be clearly seen in the fissure. The blood vessels are part of the lamellar arrangement.

Having obtained the clean surface in which the neuronal layers could be seen, a second slice 300μm was cut and this was the first slice stored. Up to five other slices of the same thickness were cut, and all six had as much entorhinal and fimbrial tissue (nomenclature of Blackstad, 1956) attached as could be cut at the same time. The slice platforms were already in the Vibratome bath and as each slice came free, with a little help from a blunt pair of forceps, it was manoeuvred on to a net with the lateral side down. Gently pushing the non-hippocampal parts of the slice against the net was sufficient to keep the slice fixed in place. Depending on the number of slices, either one or two were fixed to each net. They were fixed in the order in which they had been cut so that each slice could be identified relative to the other slices. This orientation - medial side down, fimbria to the right - meant that the electrode passed through the slice in the same track in each slice. When all the slices were cut and all the nets filled, they were loaded on to the shelves of the vertical frame in the storage bath.

2.6.4. THE BATH FOR STORING BRAIN SLICES.

Before setting up the Vibratome, the storage bath had been prepared to allow the ACSF to come to the temperature of the thermostat (33°C). The storage bath (Blaxter, 1983) is shown in FIG 2.5. It was considered advantageous to store the slices so that thay could be used one by one in the recording chamber without interfering with the remaining slices. Each slice was

FIGURE 2.5.

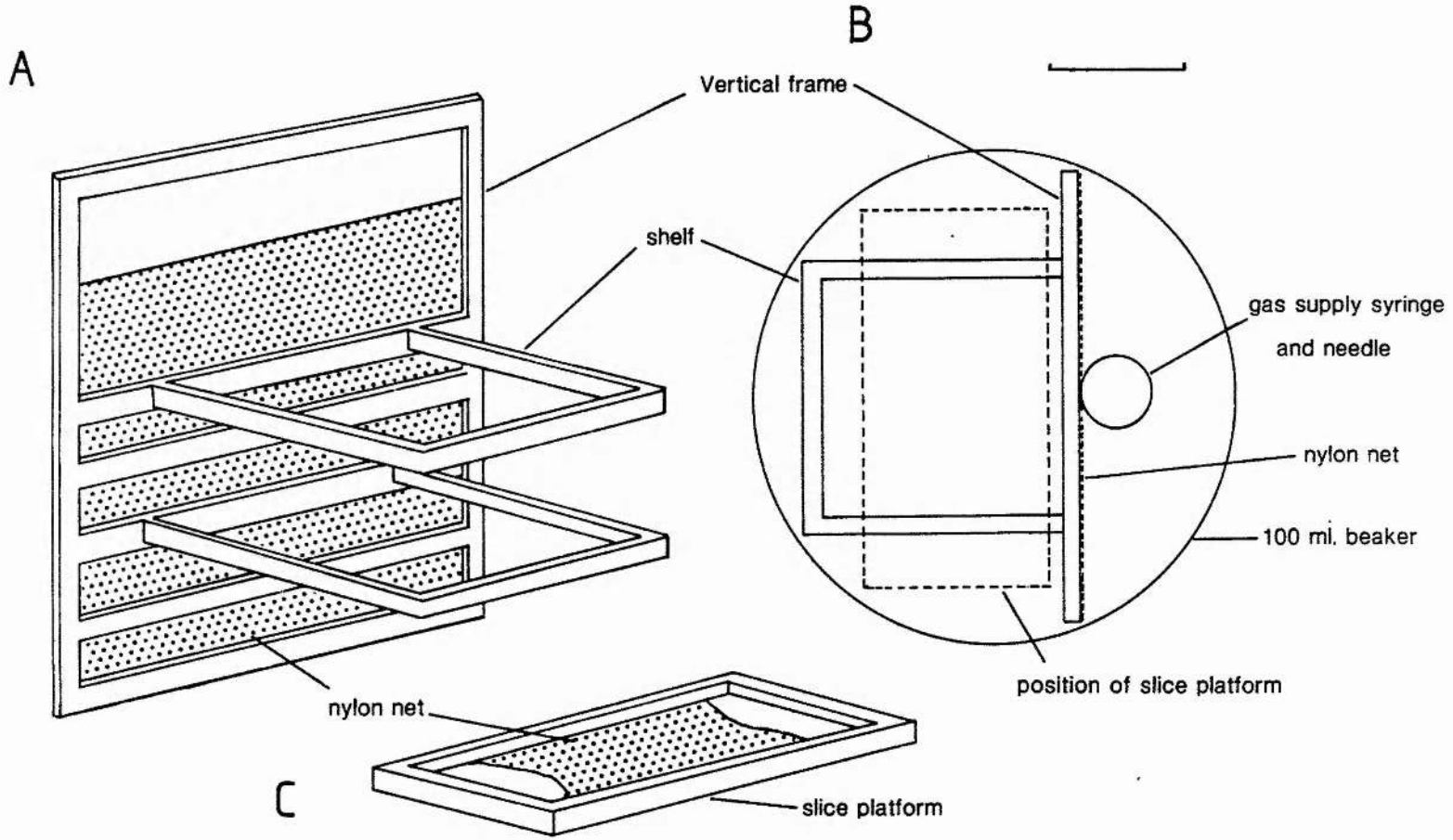
The storage system for the brain slices.

A. Perspective view of the rack of shelves. Two shelves and the gas syringe are omitted for clarity.

B. Plan of the assembly in the beaker.

C. Slice platform in the same perpective as in A.

Scale bar 1cm.


A
B
C
Vertical frame
shelf
nylon net
gas supply syringe
and needle
nylon net
100 ml. beaker
position of slice platform
nylon net
slice platform

placed on a rectangular platform, a series of which were stacked and placed in ACSF in a beaker. When required, a slice and its platform could be transferred to the recording chamber without disturbing the other slices. Gently gassing the ACSF stirs and oxygenates the saline. The slices were stored at the temperature of the recording chamber. The support shown in FIG 2.5A was made of Perspex and consists of an open vertical frame and four shelves, one above the other. To aid stirring, the Perpex parts are as small as possible. A syringe and needle were glued to the back of the frame to supply gas to the bottom of the beaker. To prevent bubbles of gas being trapped under the platforms and thus isolating the slice from the ACSF, a layer of nylon mesh from a pair of tights was glued to the vertical frame between the syringe and the shelves. FIG 2.5B is a plan view of this arrangement.

When all the slices were cut and all the nets filled, the platforms were loaded on to the vertical frame of the storage bath. Other regimes of cutting slices were tried, such as cutting the slices at room temperature or at $10^{o}C$, and storing them in ACSF at room temperature or at $10^{o}C$ but were not used routinely. The recordings were all made at $33^{o}C$.

After checking the gas supply and covering the storage bath in clingfilm, the instruments, Vibratome bath and dissecting board were washed in ethanol and hot water. No detergents were used and each razor blade was used for one

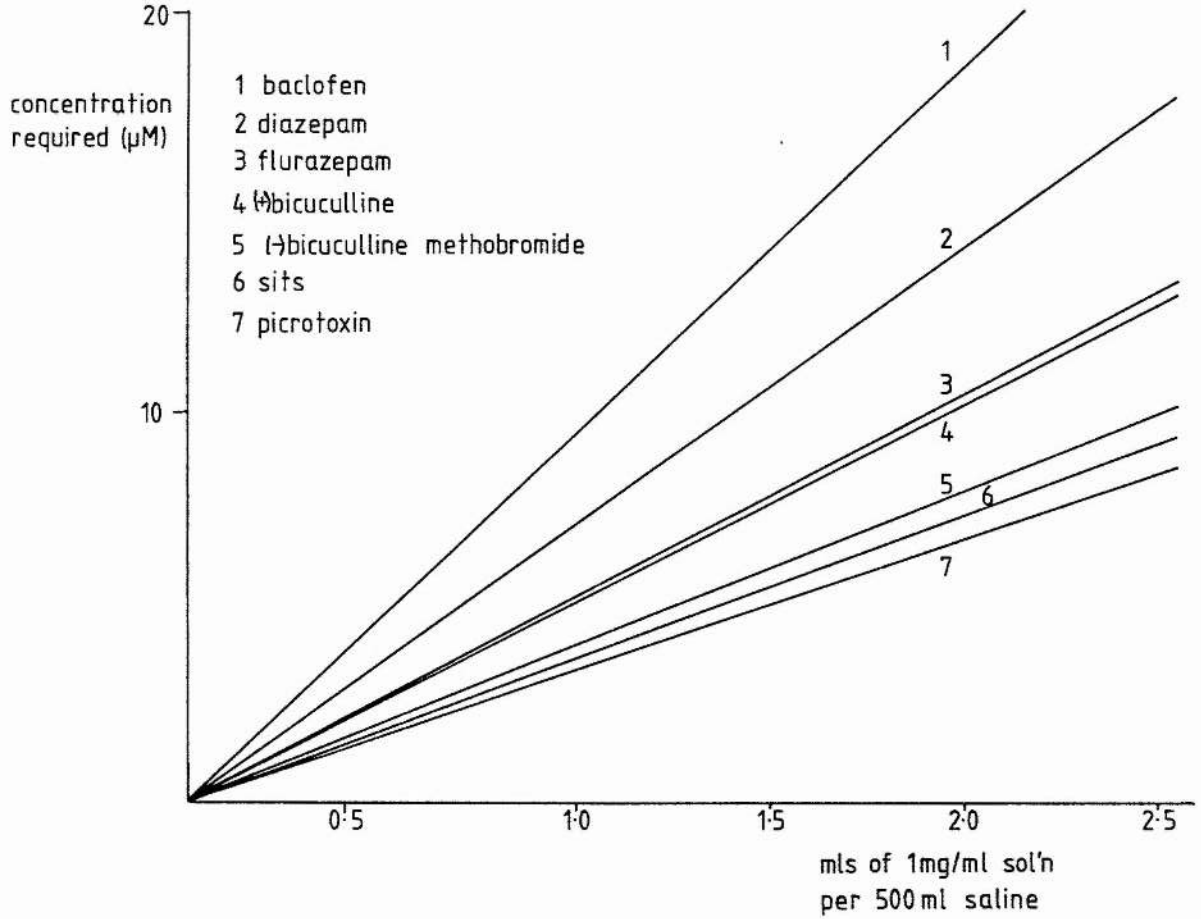
preparation only. The slices were stored for 1.5 to 2h. The time taken from the death of the animal until the slices were stored was usually 15 min, but was not less than 10min. The time taken for the dissection did not seem important to the ultimate condition of the slices.

## 2.7 DRUG SOLUTIONS.

Drugs were weighed out and dissolved in ethanol (picrotoxin, (-)bicuculline, diazepam, lorazepam, nitrazepam, clonazepam) or distilled water (flurazepam, (+)bicuculline methobromide, disodium 4-acetoamido-4′-thiocyanatostilbene-2,2′ disulphonic acid (SITS), frusemide) to give a concentration of 1 mg/ml. Enough of this solution was mixed with the appropriate ACSF to give the final desired molar concentration of the drug. The volume of drug stock required was read off from a graph that had been prepared during the design of the experiments (FIG 2.6). This method was used to minimise day-to-day errors and improve precision and accuracy. It was also quicker than weighing out exact amounts. Picrotoxin is a 1:1 molar ratio of picrotoxinin and picrotin, of which only picrotoxinin is active. Concentrations quoted are in moles of picrotoxin/litre. The active concentration is probably half that. The drug solutions were put into a reservoir and bubbled in the same way as the control ACSF.

FIGURE 2.6.

Copy of graph used to calculate the required quantity of a drug (as a solution of 1mg/ml) to give the desired final concentration in 500ml of ACSF.


20
10
concentration required (μM)
1 baclofen
2 diazepam
3 flurazepam
4 (+)bicuculline
5 (-)bicuculline methobromide
6 sits
7 picrotoxin
1
2
3
4
5
6
7
0·5
1·0
1·5
2·0
2·5
mls of 1mg/ml sol'n per 500 ml saline

## 2.8 ELECTRODES.

Intracellular electrodes were pulled from thin-walled glass (Clark Electromedical; outside diameter 1.5mm, inside diameter 1.17mm). Thin walled glass was used to maximise the amount of hole in the tip of the electrode and so improve the current-passing ability of the electrode and reduce its resistance relative to normal glass tubing. The electrode blanks were filamented to make filling rapid and complete. Electrodes were filled with filtered (>0.22μm) 2M potassium acetate 12h before use. Although this long exposure to the alkaline KAc may etch the tips (Purves 1981), the increase in strength, current-passing ability, stability and the decrease in capacitance, resistance and tip potentials made up for the loss of sharpness. Potassium chloride solution, a more ideal electrode filling solution because of the similiar ionic mobilities of the $K^+$ and $Cl^-$, was not used except in control experiments since the leak of $Cl^-$ from the electrode into the neurone interferred with the drug responses.

Four different pullers were used to pull intracellular electrodes: The first was of the Livingstone type. This had a variable heat control and delay time. The glass was pulled linearly by means of a spring attached to toothed wheels on which the glass tubing clamps were eccentrically mounted. The pull occurred as a single release of spring tension, which inevitably produced long tips up to 3cm. The tips were very

fine. Although this puller was crude compared to other newer designs, it produced excellent electrodes. Various heating coil configurations were tried. The first was a shallow loop. Most of the tip was pulled above the coil. It was hoped that this would reduce the length of the tip, but the electrodes proved more than usually non-reproducible. The second was a deep, parallel-sided coil so that all the tip would be pulled within the coil as the coil cooled. It was hoped that the tip, being smoothly cooled and protected by the coil from air currents, would be reproducible. The third variety, a 'V' shape, proved best since the tip formation occurred as the coil cooled as the glass moved up the 'V' of the coil, thus moving further away from any residual heat in the coil.

Several problems were encountered with electrodes made this way. The tips were so long and flexible that they often bent rather than pass through the slice. The position of the tip was then difficult to extrapolate from the axis of the rest of the electrode. The tapers were often curved too, because of the way the glass cooled, and the electrodes were inclined to block easily, especially as small bubbles were usually caught in the narrow parts of the taper. Many electrodes had to be pulled in order to find one sharp one.

In contrast to this, the two Ensor pullers which were used were designed to enable one to 'design' the shape of the electrode and to pull many the same after the correct setting

was found. The first Ensor was supplied by Clark Electromedical, more or less as a prototype. The horizontal optical bench system and powerful constant voltage electromagnet produced straight, short-tipped electrodes but unfortunately the six settings (length of initial pull, heater temperature, initial pull force, main pull force, coil cooling time and heat duration) had to be modified from hour to hour. A second Ensor was purchased from the new dealer, Campden Instruments. This was an extensively modified version of the original form but relied on the same principle as the first one; that a high velocity of the glass at the moment of tip separation produces sharp tips and that a two-stage pull is required to achieve this. The electromagnet was powered by a constant current power supply. As the flux produced by an electromagnet depends on the current flowing through it this makes sense, particularly as the coil heats up during use and its resistance changes. With a constant voltage supply, the current and therefore the strength of the magnet could vary with temperature. Besides this modification to the electromagnet, the optical bench had been made much more substantial and the puller settings could be varied by one part in one thousand, compared to one in one hundred of the original machine. This modification did appear necessary as changes of 1 or 2 parts per 1000 had significant effects on the tip sharpness although not on the microsopic appearance of the electrode. Both the Ensor pullers shared the particularly difficult problem that the glass tubing slipped in the chucks

which were supposed to grip it. Tightening the chucks further broke the glass. The problem was solved in four ways:

1. The glass, supplied as 15cm lengths, was cut in half and the ends fire polished by rotating them in the flame of a Bunsen burner for a few seconds. The blanks were then stored at 60°C for several days before use to relieve the stresses in the glass and make it less likely to break when compressed. The fire polishing reduced the scratching of the inside of the chuck by the sharp edges of the glass.

2. The chucks were regularly coated internally with 'Surgrip' (Fraser Chemicals Ltd, Nottingham). This is a solution of resin in ethanol usually applied to the handles of squash racquets to improve the grip.

3. Once the glass had been clamped in the chucks, a mark was made with a pen where the glass disappeared into the chucks. After pulling, slip at either chuck could be detected.

4. Campden Instruments kindly supplied a metal chuck that gripped the glass over a much wider area than the plastic chuck, reducing the force per unit area. Unfortunately, this solved the problem for the fixed side only, where the mass of the components was unimportant.

The fourth puller used was a horizontal Industrial Biosciences puller. It had the problems of the Ensor, but few of the advantages and was tried for only four pulling sessions.

The impedence of the filled electrodes (at 1kHz) was

between 25 and 100 MΩ. Sharpness was unrelated to impedance, although neurones impaled by sharp 70MΩ electrodes were often more viable than neurones impaled with a sharp 25MΩ electrode. Electrodes were checked under a microscope (400x magnification) and then filled and stored in Plasticine strips in petri dishes that were kept wet.

Iontophoretic electrodes were pulled on a vertical puller. Usually 4 electrodes were prepared for each preparation and filled with the drug solution about 2h before the experiment started. The resistance of these drug-filled electrodes was about 15MΩ.

## 2.9 IONTOPHORESIS.

The following compounds were iontophoresed: GABA, EDA, acetylcholine, glutamate and baclofen. GABA and EDA were the only two used regularly, the others being tried as easy to iontophorese (acetylcholine), to examine sodium-dependent responses (glutamate), or to look at the $GABA_B$ receptor (baclofen). Initially, GABA was used at a concentration of 1M in the iontophoretic electrode, but at this concentration leaked out of the electrode. Iontophoresis was easier with this high concentration, however, and so a compromise was reached between the ease of iontophoresis and the current artefact (see FIG 3.9 for an example of the artefact). The concentration chosen was 0.1M. This solution had a pH of 6 and so there were few $H^+$ to carry the iontophoretic current. Despite the buffering available in the ACSF, too many $H^+$ might have had an effect on the neuronal membrane. Hydrogen ions have been reported to give responses on spinal neurones (Gruol, Barker, Huang, MacDonald and Smith, 1980). The concentration of EDA in the iontophoretic elecrode was also 0.1M (pH 7) so that the iontophoretic currents of GABA and EDA could be roughly compared as doses. The transport numbers of GABA and EDA are similar (Perkins and Stone, 1982). The tips of the iontophoretic electrodes were between 1µm and 5µm diameter. With this concentration and tip size, a retaining current of less than 10nA was all that was required.

Iontophoretic electrodes often blocked, producing large switching artefacts. This reduced the reproducibility of the responses as the amount of drug ejected varied from application to application, although the iontophoretic current was kept constant by the iontophoresis programmer. The large artefacts associated with blocked electrodes were the result of switching the high DC voltages supplied by the iontophoresis programmer as it tried to maintain a constant current.

The choice of the duration of the iontophoresis and the dose cycle of the application were important because enough time had to be given to the slice to recover from the drug application. The responses reached a maximum a few seconds after the application of GABA or EDA was started, so these drugs were applied for 5s. As the recovery of the responses to GABA or EDA usually took only a few seconds after the iontophoresis was stopped 1min was considered sufficient for for the dose cycle. This combination of 5s applications and a 1 min dose cycle was established during the early experiments and proved to be useful all through the study.

One response per minute was a reasonable sampling rate; drugs took about 10min to work and ions substitutions about 5min. A shorter dose cycle would have been hard work to maintain, and a waste of chart paper, while a longer cycle might have limited the data that could have been extracted from each experiment.

## 2.10 SETTING UP THE APPARATUS.

The bath and the electronics were set up about 30min before recording was due to begin to allow the water jacket temperature to settle down, the rate of perfusion to be adjusted until stable, and to allow the equipment (especially the valve oscilloscope and the chart recorder) to warm up.

The drainage tube was placed under the lip of the bath and the suction pump started. Heater wires and the gas tube were attached to the bath and the earth (or indifferent) wire connected to the indifferent electrode in the bottom of the recording chamber. The bath was filled to within 1cm of the top with warm water. The power supply and temperature controller were then switched on with the rest of the equipment in the rack and the gas supply regulated so that about 100 ml/min flowed through the water jacket and into the recording chamber. One of the perfusion bottles was filled with the normal ACSF, the other with whatever modified ACSF was used. The tubing to the bath and to waste was cleared with pressure from a syringe so that the flow of ACSF was minimally interrupted during the changeover to another solution. When the ACSF was flowing smoothly, the flow was switched into the recording chamber and reduced to 1 - 3 ml/min (ie 1 drop every 1 - 3 s). The piece of nylon mesh that carried the used ACSF over the lip of the recording chamber to waste was wetted and placed in position on the floor of the chamber. The air holes

from the water jacket were cleared of water with a dry pipecleaner to ensure that the slices did not dry out.

## 2.11 EVALUATION OF THE SLICES.

A slice platform was removed from the storage bath and placed in the recording chamber with the inlet tube for the ACSF inside the frame of the platform. The lid was placed as far as possible over the slices as was consistent with accessibility of the electrodes. The rate of perfusion was checked and the light adjusted to illuminate the slice at a convenient angle. The slices were examined with the low power of the zoom microscope and their viability determined. The following points were considered. Typically, slices 300μm thick were translucent white with clear grey pyramidal neurone and granule neurone layers. Pyramidal neurone layers in good slices extended up to the entorhinal cortex at one end and around the slice into the V-shape of the granule neurone layer at the other. Thin slices allowed the underlying net to be seen through them and sank into the net rapidly, distorting the slice surface and cutting off the perfusion under the slices. Thick slices did not to have well defined edges, but flopped down round the alveus, rounding the upper surface. This rounding was easily detected by the way light reflected off the slice. The neuronal layers were distorted in thick slices and often not clearly defined, making it difficult to judge the position of the neurone that had been impaled. There may also have been a lack of oxygen in the centre of the slice. Drugs took up to 25 min to act in thick slices.

## 2.12 IMPALEMENT OF PYRAMIDAL NEURONES.

Usually one of the slices on the net was suitable. Slices were used in the reverse order in which they were cut since the later slices were viable more frequently.

Once the slice had been selected, an iontophoretic electrode was placed in the holder of a Prior micromanipulator and positioned under low power observation near the entorhinal end of the slice. An intracellular electrode was placed in the hydraulic manipulator and positioned just basal side of the pyramidal neurone layer.

At this point, the DC preamplifier was switched on and the electrode advanced until a trace appeared on the oscilloscope screen, signalling that the electrode was in the slice. The perfusion was checked again and the impaling sequence begun.

After placing the electrode in the slice on the basal side of the CA1 pyramidal neurone layer, the impedence of the electrode was measured. The bridge was balanced by passing 100ms pulses at 1 Hz through the electrode and changing the balance potentiometer until the deflection disappeared, leaving the capacitance artefact. This was reduced as far as possible with the capacity compensation potentiometer. The bridge was usually then slightly unbalanced so that a negative deflection was just visible. The electrode was advanced through the

slice. When its tip touched a neurone membrane, the impedance increased and the downward deflection became larger, often doubling or more in size. This was usually accompanied by a change in the DC level of the signal as the electrode detected the potential of the neurone near its tip. Positive (upward) potentials were presumably recorded from the extracellular layer near the cell membrane, and negative deflections were presumably partial impalements. As soon as these events occurred, one of three of the following was done:

1. A short pulse (as short as could be switched manually) of positive current (10 - 100nA) was passed through the electrode.

2. The electrode was "whistled" with the capacity oscillation button on the preamplifier.

3. The capacity compensation potentiometer was turned until the system oscillated.

The last two were used little. Oscillation of the electrode not only produced electrical buzz but also mechanical movements of the electrode. This was sometimes useful for forcing an imperfect electrode into a neurone. The best impalements were made with the current pulse; the positive tip attracted the neuronal membrane to it fast enough for the electrode to pass into the neurone.

All three methods of impalement often changed the impedence of the electrode and thus may have affected tip potentials. The membrane potential ($V_m$) was measured on

withdrawing the electrode from the neurone and any changes induced earlier by intracellular injection of current or the application of a drug was related to this value of $V_m$. Potentials recorded when the electrode was still in the neurone are referred to as membrane potentials. The value of the membrane potential includes the changes in tip and junction potentials between the inside and the outside of the neurone. When a neurone was penetrated with a good electrode, a DC potential of about -40mV accompanied by numerous spikes between 40 and 70 mV high were seen on the oscilloscope screen. At this point, the oscilloscope sweep speed was increased from 2 s/cm to 50ms/cm with a X10 multiplier to enable the duration of the spike to be measured and to allow the bridge to be rebalanced if necessary. The sweeps of the oscillioscope were triggered by the hyperpolarizing pulses from the stimulator. The bridge was roughly balanced by removing the voltage transients from the hyperpolarizing pulse with the balance potentiometer. After 30s or so, the membrane potential increased to about -60 mV and the spikes became less frequent and larger. In the next minute, the duration of the spikes decreased to 1 - 2 ms and the input resistance rose. The neurone was then left until the membrane potential stabilised. The capacity compensation was turned up, until the system was on the verge of oscillation. This removed the capacity transients from the hyperpolarizing pulse and allowed the bridge balance to be finely adjusted.

## 2.13. EVALUATION OF THE PYRAMIDAL NEURONE.

Many criteria have been used to define a viable pyramidal neurone. Most of them were used here, as they were all easy to look at.

1. Membrane potential was more negative than -55mV and varied little (<3mV in the experiment).

2. Spike duration was less than 2 ms (measured at the baseline).

3. Spike amplitude overshot 0 mV by at least 10 mV.

4. Depolarizing pulses of 10 mV or a little less produced spikes (the number depending on the amount of current injected) but not immediately after the onset of the pulse.

5. Input resistance (defined here as the voltage deflection in mV produced by an intracellular hyperpolarizing current injection of 1 nA) was 8 MΩ or more. neurones with an input resistance of less than 15MΩ were used only if all the other parameters were satisfactory.

6. Membrane time constant was about 10-15 ms and was easily separable from the capacity transients of an unbalanced electrode.

7. Spike trains initiated by depolarizing pulses (100 ms duration at 1 Hz) were followed by a hyperpolarization of the membrane and an increase in conductance).

8. neurones responded to GABA and recovered quickly from exposure to the drug (this may have measured processes in the slice other than the ability of the neurone to maintain ion

gradients).

9. The impalement was able to withstand capacity overcompensation and 10nA of depolarizing current. This gave some idea of how well the neurone is impaled. If an impalement survived this, it was rarely lost during the experiment.

If the neurone was viable, then the electrode balance was checked again and the size of the hyperpolarizing pulses adjusted to give a deflection of 5 - 7 mV on the chart recorder. These pulses were used to measure conductance changes, since they were produced by constant current pulses from the stimulator. The voltage deflection which a constant current produces is, by Ohm`s Law, proportional to the resistance that the current is flowing through, in this case the input resistance of the neurone. Small pulses were used to reduce interference from voltage-dependent conductances.

## 2.14 EXPERIMENTAL PROTOCOL.

Drugs were locally applied by iontophoresis. The track of the iontophoresis electrode was adjusted to pass through the dendrites of the impaled neurone or through the cell body region. The iontophoresis programmer, under manual control, was set to give 10nA retention current and 40nA ejection current. With the batteries switched on, the electrode was placed near the surface of the slice and with the fine control of the manipulator advanced a little way into the slice. The contact with the slice was seen as an artefact on the chart recorder. The ejection current was not switched on until the electrode was in the slice because it tended to produce a gout of drug at the moment of contact with the slice. The electrode was advanced through the slice until a response was seen and then the ejection current switched off for a minute, when the drug was applied again for 5s. If the response occurred within 1s of the ejection current being switched on and if the time course, recovery, conductance change and artefact were satisfactorily typical, the electrode was left where it was. Otherwise it was moved around until the desired response was found (typically 50% conductance increase, 7 mV for the depolarizing response and 60%, 4 mV for the hyperpolarizing response). Frequently the response was contaminated by the current artefact (eg see FIG 3.9), especially when the iontophoretic electrode was blocked or blocking. If the response proved to be constant for a few minutes and the

artefact was not due to blocking of the electrode, then the experiment continued. If electrode blockage could not be cleared by current pulses, then the electrode was changed. With care it was possible to do this without losing the impalement.

When satisfactory responses were obtained, the dose cycle was started. GABA or EDA were applied for 5s every min. The 5s was timed by the stimulator and the minute by a mechanical clock. Initially the ejection current was set at 40nA or less if a half maximal response could be so obtained. The retention current was reduced to as far as possible consistent with low drug leakage from the electrode tip. As large tipped electrodes were used, low ejection currents were required. Initially the amount of GABA ejected from the tip varied becuse of dilution produced by bulk flow of ions and water into the electrode during the retain phase of the cycle but eventually an equilibrium was reached between the diffusion of the drug out of the iontophoretic electrode and the dilution produced by the retention current.

Dose response curves (DRCs) were obtained starting from low ejection currents (low doses) and ascending since high ejection currents (high doses) produced desensitization of subsequent applications. Sometimes a DRC was unnecessary but one ejection current was used throughout.

Towards the end of the study, it became necessary to obtain hyperpolarizing responses that were not mixed with depolarizing responses. Consequently, a method that may have reduced the spread of the iontophoresed drug was used. A large (400nA) ejection current was preset and this turned on and off, not for the usual 5s, but for 10ms to 100ms. These small bursts of GABA produced rapid, uncontaminated responses.

After obtaining reproducible responses at, typically, 40nA, a DRC was made starting at low ejection currents and ascending to high ejection currents. Then the reproducibility of the responses was checked with more applications at the original ejection current. If the reproducibility was satisfactory, the normal ACSF was changed to the next one required, which had been running through the tap to waste. Since the tubing clamp was between the tap and the recording chamber, the new solution flowed at the same rate as the old. The differences in heights of the reservoir had no effect on this rate. Drug applications were continued and closely watched until the responses had changed to their new level, when washout with normal ACSF was started. The exception to this design was when the threshold concentrations of picrotoxin and bicuculline were being measured. Washout was then started when a significant effect on the response was seen. Drugs typically took 10 - 20 min to influence the impaled neurone, while ions took 3 - 10 min. The reproducibility of responses just after a solution change compared with just before a

solution change was particularly noted in order to be be sure that the change itself had had no effect on the slice. Responses were recorded on chart paper usually at 1mm/s and 5mm/s. Important responses (ie those during DRCs and solution changes) were recorded at the faster speed. Notes on speed of chart, amplification, time, solution changes, input resistance, spike amplitude and duration and, at the end, the $V_m$ were written directly onto the chart paper in red pen which could be removed with a red filter, making photography of prepared figures more simple.

## 2.15 ANALYSIS OF DATA.

This took four forms:

1. Direct comparison of the voltage amplitude or conductance change associated with a response. Usually the reproducible controls were compared with the responses in a drug solution or an ion substituted solution and the washout responses compared with controls.

2. For experiments where DRCs were made, the responses (either as amplitudes or conductance changes) were plotted against the iontophoretic currents which produced them. The responses in the drug solution and the responses in the washout were plotted alongside.

3. The results from experiments involving intracellular current injection were plotted either as current/voltage curves or as response amplitude against membrane potential.

4. The reversal potential of responses could be gained also by a graphical method.

Following the theoretical outline of Ginsborg (1967), Assaf, Crunelli and Kelly (1981) produced an equation which they used to find the reversal potential for a depolarizing response to GABA and 5HT in hippocampal granule neurones, viz

$$V = (E_{rev} - V_m).(1 - R_m^*/R_m)$$

where V is the depolarization produced by GABA, $V_m$ is the membrane potential, $E_{rev}$ is the reversal potential for the

response, $R_m^*$ is the input resistance during application of GABA and $R_m$ is the resting input resistance.

This is the equation for a straight line, with $E_{rev}$ being the Y axis intercept. This method yielded a reversal potential of -40mV in a neurone with a resting membrane potential of -60mV (Assaf et al 1981). Williams, Egan and North (1982) used the same method to determine the reversal potential for responses to enkephalin in locus coerulus neurones. In both these experiments, the method was applied to responses of the cell body, very close to the site of impalement. Any problems arising from the electrotonic properties of the neurone were therefore minimised (see Discussion). The same method has been used here to estimate reversal potentials for a response in the dendrites of pyramidal neurones. The method has been extended a little to determine whether the responses are entirely mediated by chloride ions. Changes in $E_{Cl}$ produced by a change in $[Cl^-]_o$ can be calculated without knowing $[Cl^-]_i$. A change of $E_{Cl}$ of 10mV was chosen in these experiments for two reasons. First, this usually produced a negligible change in the ground potential and secondly, it had little effect on the input resistance of the neurone (FIG 3.18). The change in chloride concentration required is a reduction from 130.5mM to 88mM, substituting the impermeant anion isethionate. The reversal potential was measured in normal saline by contructing a dose response curve for GABA iontophoresed onto the stratum radiatum dendrites. The reversal potential method is independent of actual drug

concentration. The size of the response was plotted against the conductance ratio (ie the size of the constant current hyperpolarizing pulse during drug application divided by the control size of the constant current hyperpolarizing pulse). The same dose response curve was obtained again when the slice had equilibrated with the low chloride saline. If the $E_{rev}$ of the response had changed by 10mV, then the response must have been entirely due to a change in chloride conductance, unless the low $Cl^-$ ACSF had an effect on the equilibrium potential for an other ion.

# CHAPTER THREE

# RESULTS

This chapter is divided into 5 sections;

1. Properties of the pyramidal neurones.
2. Responses of the pyramidal neurones to acetylcholine and glutamate.
3. Responses of the pyramidal neurones to GABA and EDA.
4. Effect of ion substitution on the responses to glutamate, GABA and EDA.
5. Pharmacology of the responses of the pyramidal neurones to GABA and EDA.

Results are taken from 272 neurones that were successfully impaled. Bars under the responses shown in the figures give the duration of iontophoresis. The total length of the diagrams showing the position of iontophoresis represents about 1mm.

## 3.1. PROPERTIES OF THE PYRAMIDAL NEURONES.

A sample of the neurones used in the experiments had a mean membrane potential ($V_m$) of -63.5mV (n = 107), a mean spike amplitude of 77.5mV (n = 50), a spike duration of less than 2ms (measured at the baseline) and an input resistance (1nA cathodal current) of 15M$\Omega$ (n = 44). The distribution of these parameters is shown in FIG 3.1. These values relate to neurones immersed in saline with 4mM $Ca^{++}$ or the usual 2mM $Ca^{++}$. The former concentration was used initially but on changing to the 2mM concentration, no difference was observed in the viability of the neurone. It was thought that the higher concentration of $Ca^{++}$ might stabilize the neuronal membrane and help it to seal more quickly around the electrode in the few minutes after impalement.

The action potentials of all the neurones used in experiments overshot 0mV. It was common to find neurones which, probably due to damage, had low resting potentials and small, slow action potentials (up to 50ms long). Such neurones were not used in experiments. neurones at the top and bottom of the slice were avoided since they may have been damaged.

All the neurones used in experiments were thought to be CA1 pyramidal neurones because:

1. Impalements of neurones smaller than pyramidal neurones were unstable. Granule neurone impalements lasted no

FIGURE 3.1.

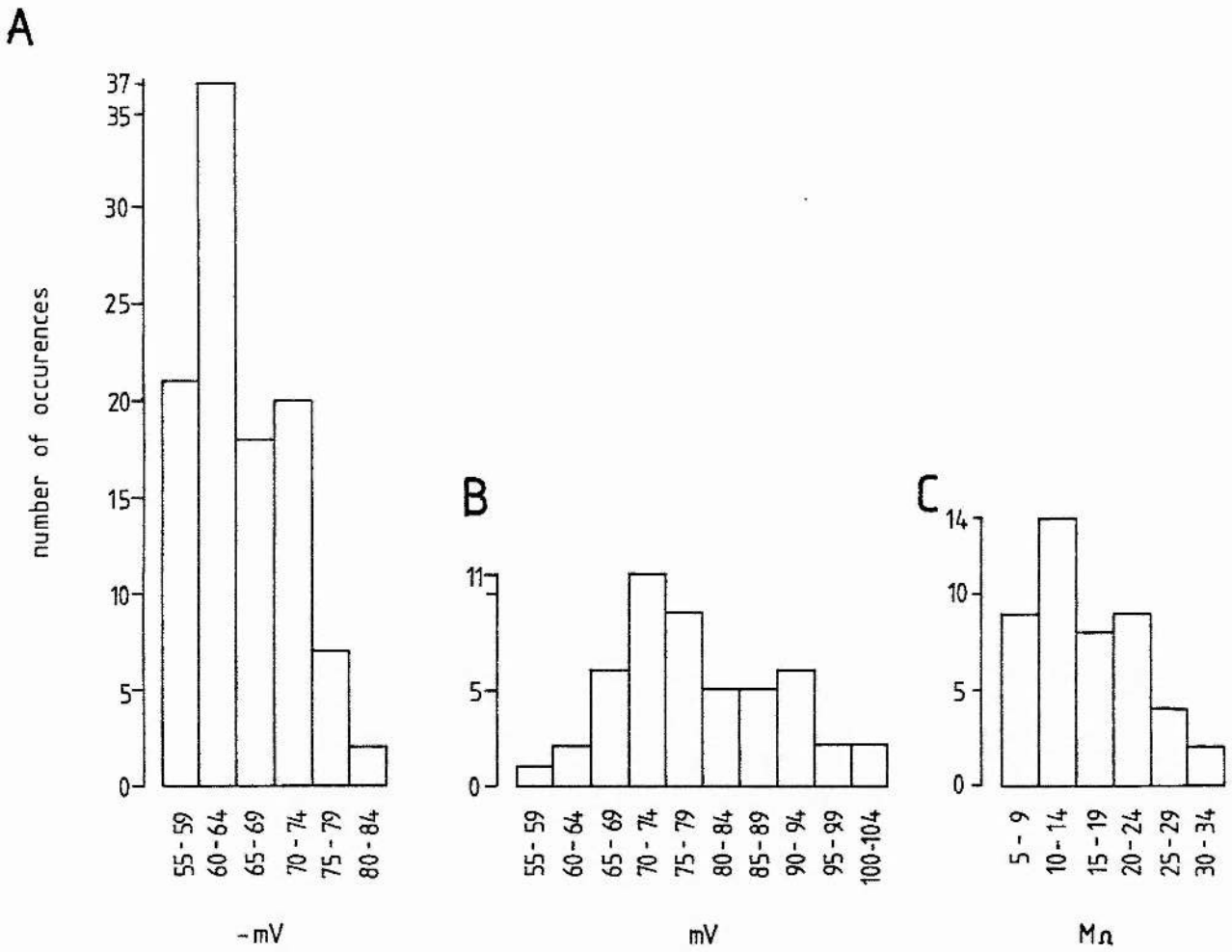
The distribution of:

A. membrane potential,

B. spike amplitude,

and C. input resistance

of a sample of 107 cells.


A
number of occurences
37
35
30
25
20
15
10
5
0
55 - 59
60 - 64
65 - 69
70 - 74
75 - 79
80 - 84
- mV
B
11
5
0
55 - 59
60 - 64
65 - 69
70 - 74
75 - 79
80 - 84
85 - 89
90 - 94
95 - 99
100-104
mV
C
14
10
5
0
5 - 9
10 - 14
15 - 19
20 - 24
25 - 29
30 - 34
MΩ

more than a few minutes. Granule neurones are the same size as the non-pyramidal neurones which are found in the CA1 region of the slice.

2. In a few experiments the pathways intrinsic to the slice were stimulated with monopolar tungsten electrodes and the intracellularly potentials thus evoked were recorded. FIG 3.2.1 shows that stimulation of the excitatory input from the CA3 neurones to the stratum radiatum (Schaffer collaterals) produced EPSPs and spikes in the pyramidal neurone and that hyperpolarizing potentials could be obtained by stimulating the basket neurones via antidromic stimulation of the pyramidal neurones. A spontaneous IPSP at a fast sweep speed is shown for comparison (FIG 3.2.3). This provides evidence not only that the neurones were pyramidal neurones but that some of the pathways were intact, including the recurrent inhibitory pathway of the pyramidal neurones which runs laterally as well as lamellarly in the hippocampus.

3. The neurone fired following depolarization by intracellular current injection. Immediately after the depolarization was stopped, the membrane potential became more negative than the baseline and the membrane conductance increased. These changes in the neuronal membrane may have been produced by recurrent inhibition or a voltage-dependent conductance that is known to be present in these neurones (Haas and Konnerth, 1983). The effect of depolarization of the neurone is shown in FIG 3.3.

4. On two occasions, partly to test the slicing technique

FIGURE 3.2.

Stimulation of the slice.

1. IPSPs produced by stimulation of the alveus (30V, 0.5ms, 2Hz.).

   $V_m$ -70mV.

   Scale bars 5mV, 400ms.

2. EPSPs from stimulation of the Schaeffer colaterals (30V, 0.5ms, 2Hz.).

   Note spikes on some of the EPSPs.

   $V_m$ -70mV.

   Scale bars 5mV, 400ms.

3. Spontaneous IPSP.

   $V_m$ -55mV.

   Scale bars 5mV, 80ms.

Note different calibrations.

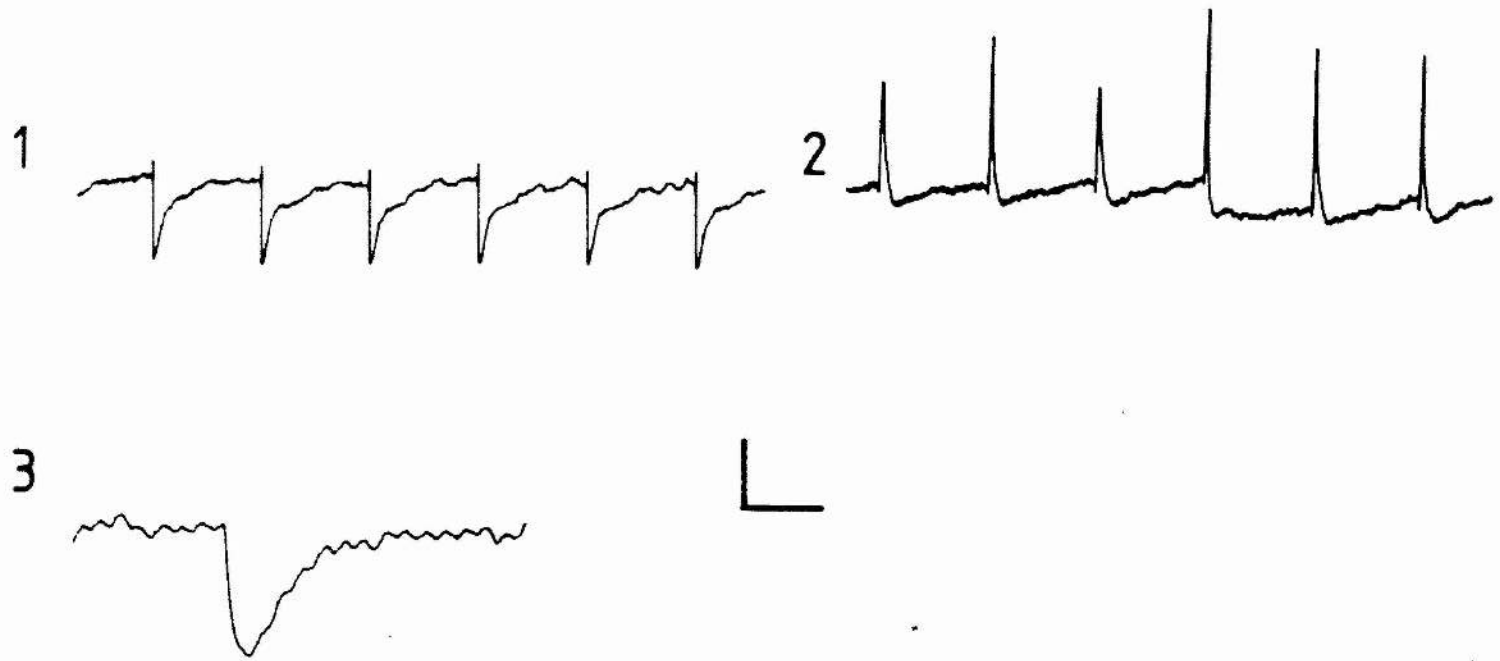

1
2
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FIGURE 3.3.

Intracellular stimulation produces what may be recurrent inhibition.

1. 20mV depolarization for 0.6s produces 20 spikes and a hyperpolarization of the neurone accompanied by an increase in membrane conductance.

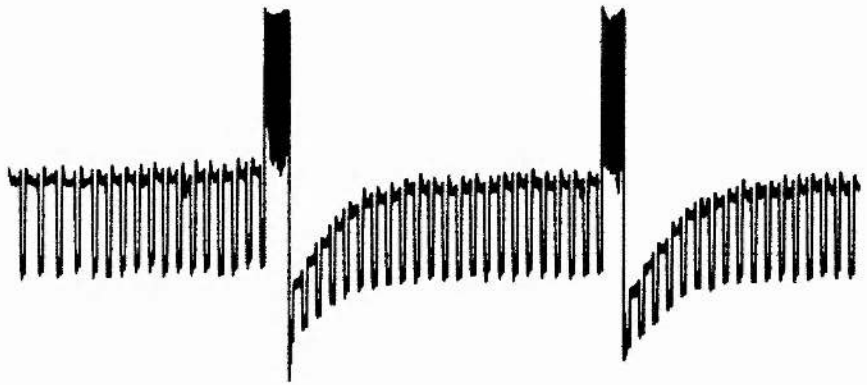
   Scale bars 5mV, 2s.

2. The same stimulation recorded at a faster sweep speed.

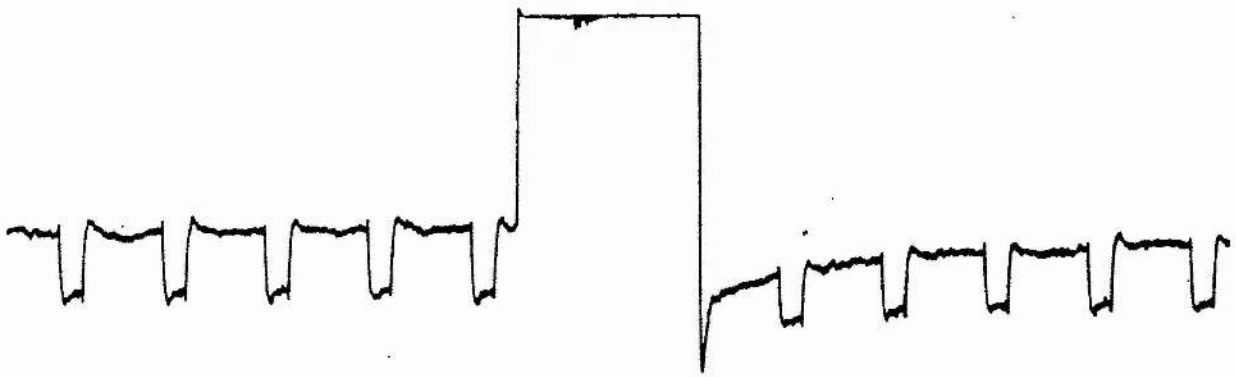
   Scale bars 5mV, 400ms.

$V_m$ -60mV.

1



2



and partly to evaluate the impaled neurones, electrodes of normal configuration and sharpness were filled with Lucifer yellow dye. This was injected into any neurones impaled. It was assumed that the electrodes penetrated pyramidal neurones. The slice was then removed from the recording chamber, fixed in 4% formalin and then cleared in methylsalicylate. Under the fluorescence microscope, the filled neurones appeared to be CA1 neurones similar to those described by Turner and Schwartzkroin (1980) and by Cobbett and Cottrell, (1981).

5. The prepotential of the spike, as observed by Kandel et al (1961) in pyramidal neurones in vivo was sometimes seen in this study, especially during the initial stages of the impalement. The prepotential was only separated from the main spike by a small step and change of slope.

Only a few neurones in this study fired action potentials regularly throughout the experiment. Small amplitude spikes appeared often, however, and these were much faster than synaptic potentials. Perfusing the slice with low $K^+$ ACSF produced a hyperpolarization always much less than that expected from calculations made with the Nernst equation for the changes in the concentration of potassium.

## 3.2. RESPONSES OF THE PYRAMIDAL NEURONES TO ACETYLCHOLINE AND GLUTAMATE.

### 3.2.1 RESPONSES OF THE NEURONES TO ACETYLCHOLINE.

This drug was used in some early experiments because it was known to be easily iontophoresed. Responses were easy to obtain and tested the method of preparation and the viability of a slice and the neurones in it. Acetylcholine produced depolarizations which were excitatory. The responses were accompanied by an apparent decrease in conductance.

### 3.2.2. RESPONSES OF THE NEURONES TO GLUTAMATE.

Glutamate was used here because some of its actions are due to an increase in $Na^+$ conductance. As solutions with zero or very low [$Na^+$] affected the viability of neurones, only moderate substitutions of $Na^+$ could be made. It was necessary to test whether the changes in $E_{Na}$ that resulted from this level of substitution were sufficient to affect the size of $Na^+$-dependent responses. Since there were problems selecting a substituent cation for $Na^+$ in experiments on the ionic mechanism of GABA and EDA responses, it was decided to test the effects of various substituents on a known $Na^+$-dependent response. Responses of the neurones to glutamate were small compared with the difference between the membrane potential and $E_{Na}$.

Although glutamate responses were readily found, the underlying conductance changes were complex, there being a decrease as well as an increase in conductance. The two responses were not separated by moving the position of the iontophoretic electrode. The substitution of all the NaCl with sucrose, leaving 26mM $Na^+$, had variable effects on the responses. A pure $Na^+$ dependent response not being found, the experiments were discontinued.

FIGURE 3.4.

Responses of the dendrites to GABA and EDA.

1. EDA iontophoresis (50nA).

   $V_m$ -60mV.

Note that the response is biphasic.

2. GABA iontophoresis (60nA).

   $V_m$ -55mV.

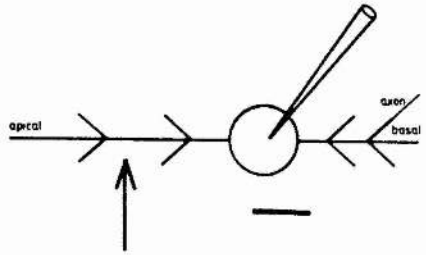
3. GABA iontophoresis (30nA).

   $V_m$ -70mV.

Note that the response is biphasic.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis. Scale bar represents 100μm.


apical
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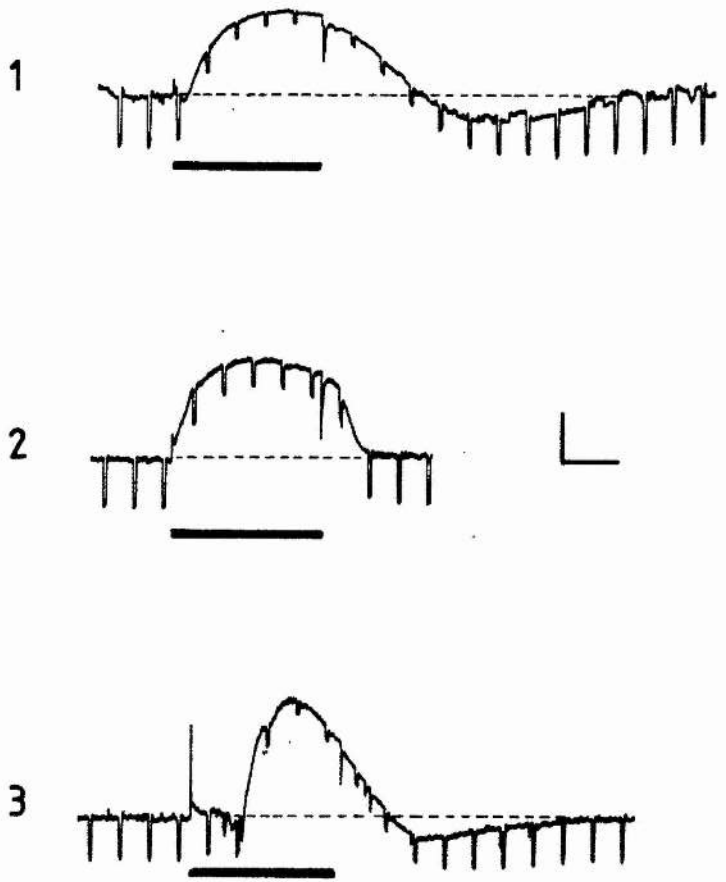
basal


1
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3

## 3.3 RESPONSES OF THE PYRAMIDAL NEURONES TO GABA AND EDA.

### 3.3.1. RESPONSES OF THE DENDRITES.

#### 3.3.1.1. DEPOLARIZING RESPONSES OF THE DENDRITES.

GABA applied to the dendrites produced a depolarization (FIG 3.4.2 and 3.4.3). Such a response was present throughout the dendritic tree. The response was found in all the neurones tested, and was accompanied by an apparent increase in conductance. In one experiment, GABA was applied to different parts of the dendritic tree at a constant iontophoretic current. When applied near the cell body, the depolarizing response of the dendrites was large and accompanied by a 66% increase in conductance. When applied to the stratum radiatum, the response was equally large but accompanied by a 30% increase in conductance. When applied to the dendites near the fissure, only a small depolarizing response could be found. This was accompanied by an increase in conductance of only 10%. The responses are shown in FIG 3.5A. If the iontophoretic current was varied in order to obtain depolarizing responses of the same size from all three regions, the response near the cell body was accompanied by the largest increase in conductance and the response near the fissure was accompanied by the smallest increase in conductance. The apparent changes in conductance may have been distorted by the electrotonic parameters of the neurone (see introduction).

FIGURE 3.5.

Depolarizing responses to GABA obtained at different sites in the dendrites.

1. GABA iontophoresis (50nA).

   A. Response of the dendrites obtained near the cell body.

   B. Response of the dendrites in the stratum radiatum region.

   C. Response of the dendrites obtained near the fissure.

2. Responses of the same size obtained by varying the iontophoretic current.

   A. Response obtained near the cell body.

   B. Response of the dendrites in the stratum radiatum region.

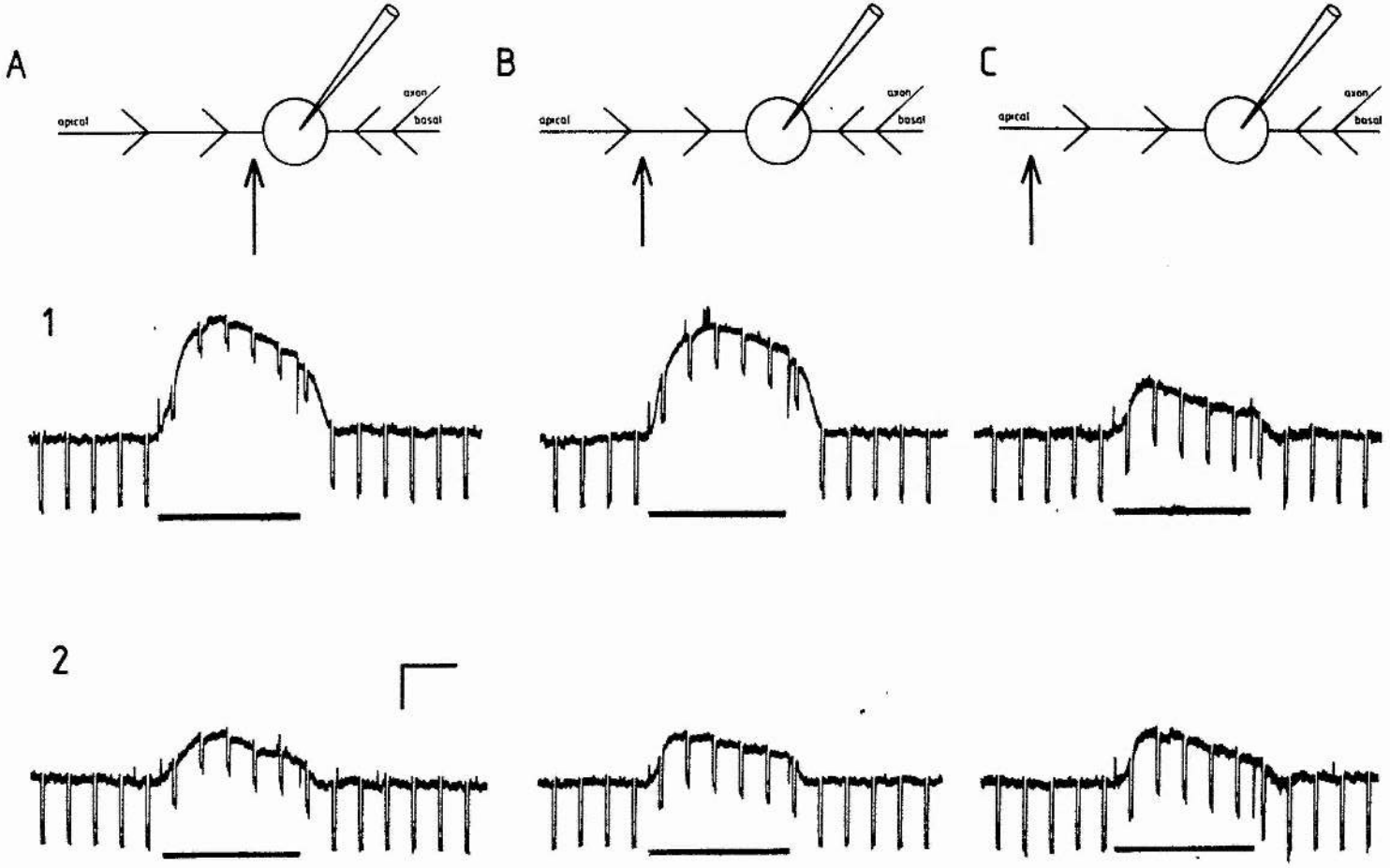
   C. Response of the dendrites obtained near the fissure.

$V_m$ -65mV.

Scale bars 5mV, 2s.

Note the differences in size and conductance change of the responses.

Diagrams show the positions of iontophoresis.


A
B
C
apical
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basal
1
2

During a 5s application of GABA, the response reached its peak after about 2s and then decreased for the remaining 3s to a level just above the baseline. The membrane potential and the increase in conductance returned to resting levels within 3s after the end of the 5s application period. If the application of GABA was continued for 10s, then the response decreased to the baseline. Spikes could sometimes be seen on very large responses (FIG 3.6).

Iontophoresis of hydrochloric acid (0.001M, pH=3) onto the neurone had no effect that resembled any of the responses of the neurone to GABA or EDA. Presure application of GABA in low concentrations (100μM) produced responses of the neurone that resembled the responses produced by the iontophoresis of GABA. The responses of the neurone to GABA or EDA do not appear to be artefacts.

The size of the response was reduced by withdrawing the iontophoresis electrode 20μm, suggesting that the diffusion of GABA was localized.

Although the responses decreased in size during the application of GABA, it was possible to obtain reproducible responses with a 1 min dose cycle. If the interval between applications of GABA was less than this, then the dendrites desensitized. The depolarizing part of a biphasic response disappeared when GABA was reapplied 15s after the first

FIGURE 3.6.

Three consectutive depolarizing responses of the dendrites showing that action potentials can appear during a normally inhibitory response.

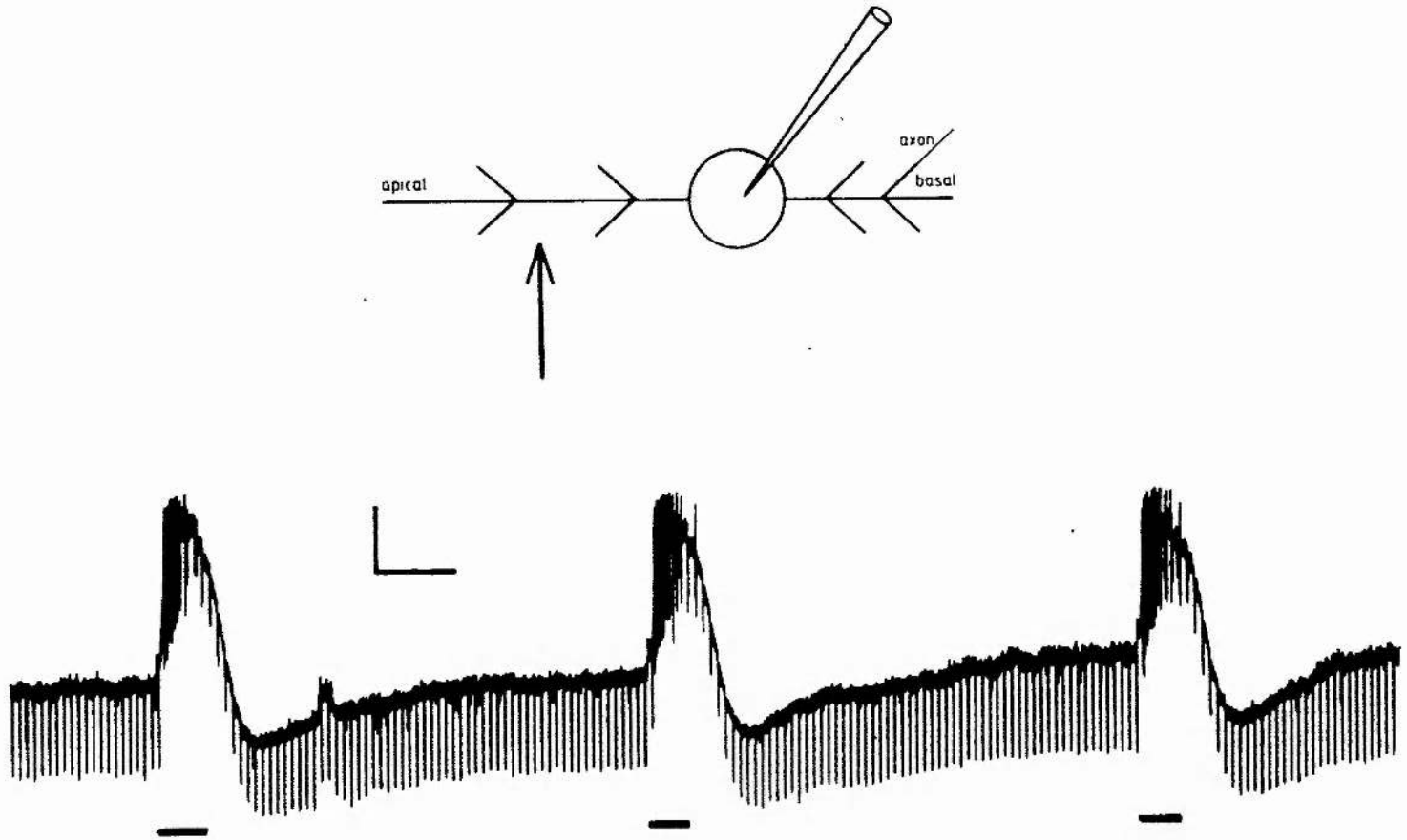
EDA iontophoresis (65nA) in the presence of diazepam (10uM).

$V_m$ -58mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

The chart recorder was unable to follow the spikes. The spikes had the appearance of full action potentials.


apical
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application of GABA (FIG 3.7). Even responses obtained at 1 min intervals, however, may have been desensitized, because stopping the application of GABA for a few minutes occasionally had the effect of increasing the size of the response when GABA was reapplied (FIG 3.8). To avoid this problem, low ejection currents of GABA were used where possible and the responses checked for desensitization. Depolarizing responses of the dendrites to GABA obtained near the cell body were increased in size when the cell body was hyperpolarized but the size of responses obtained further away on the dendrites were not changed by hyperpolarization of the cell body.

EDA produced a similar depolarizing response of the dendrites with respect to time course, recovery rate, reversal potential and desensitization. Depolarizing responses of the dendrites to EDA obtained near the cell body were increased in size when the cell body was hyperpolarized (FIG 3.9).

In an attempt to detect any synaptic contribution to the depolarizing response of the dendrites, four slices were perfused with low $Ca^{++}$ / high $Mg^{++}$ ACSF. There was no effect on responses to either GABA or EDA. FIG 3.10 shows a control EDA response and the response in the modified ACSF. Low $Ca^{++}$ / High $Mg^{++}$ ACSF blocked synaptic potentials produced by stimulation of the impaled neurone either antidromically or orthodromically with tungsten electrodes (stimulus was 5-10V, 100μs, at 0.1Hz.). Perfusion of the slice

FIGURE 3.7.

Separation of two different responses by desensitization.

GABA iontophoresis (2nA).

The first response was a cell body hyperpolarization with a depolarization superimposed. 15s after the first application of GABA a second application of GABA was made. The depolarization was smaller and the hyperpolarization consequently more obvious.

$V_m$ -70mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

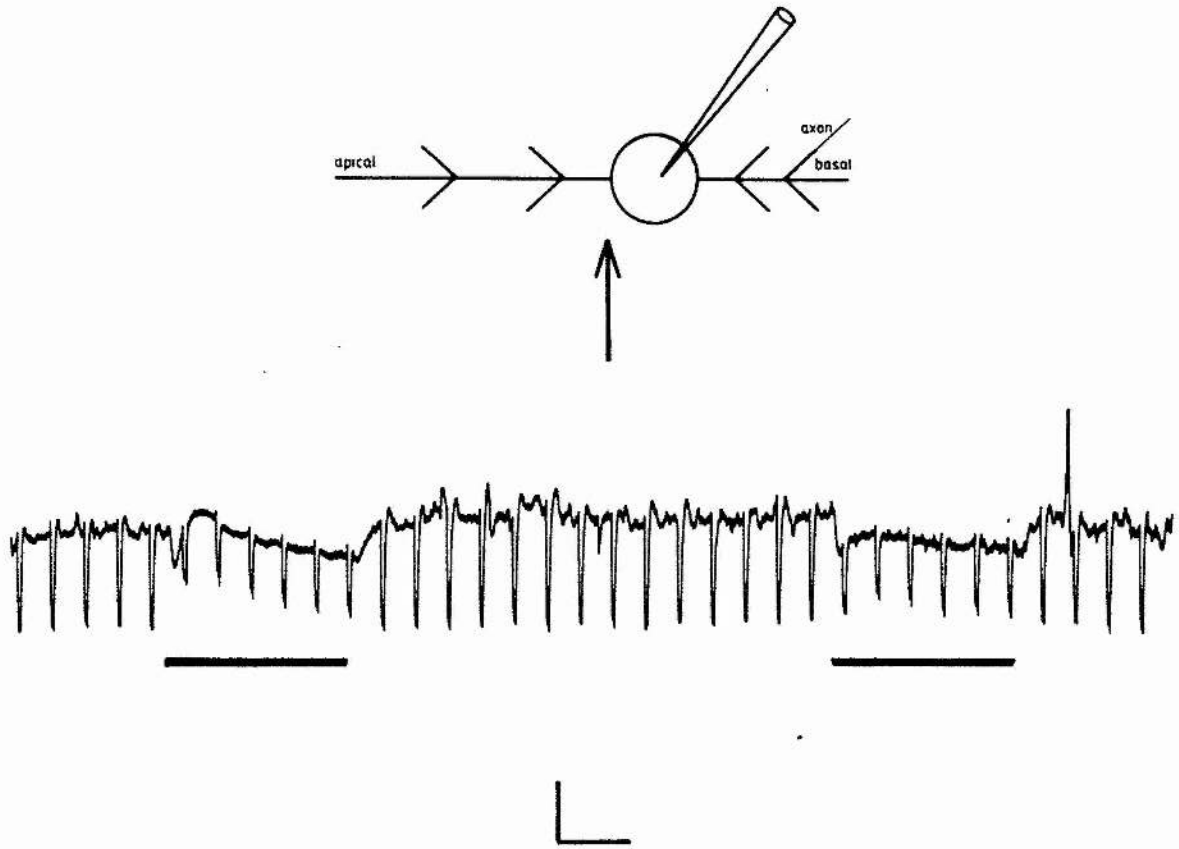

apical
axon
basal

FIGURE 3.8.

Effect of flurazepam (10μM), diazepam (10μM) and periods without iontophoresis on the size of depolarizing responses of the dendrites. Y axis; response size in mV. X axis; minutes from start of experiment.

Arrows show the times when the tap controlling the solutions was switched.

GABA iontophoresis (40nA).

1. flurazepam.
2. diazepam.
3. no GABA application.
4. no GABA application.

$V_m$ -55mV.

Note that the action of flurazepam and diazepam are opposite and the increase in size of the responses to GABA after periods with no applications of GABA.

Diagram shows the position of iontophoresis.

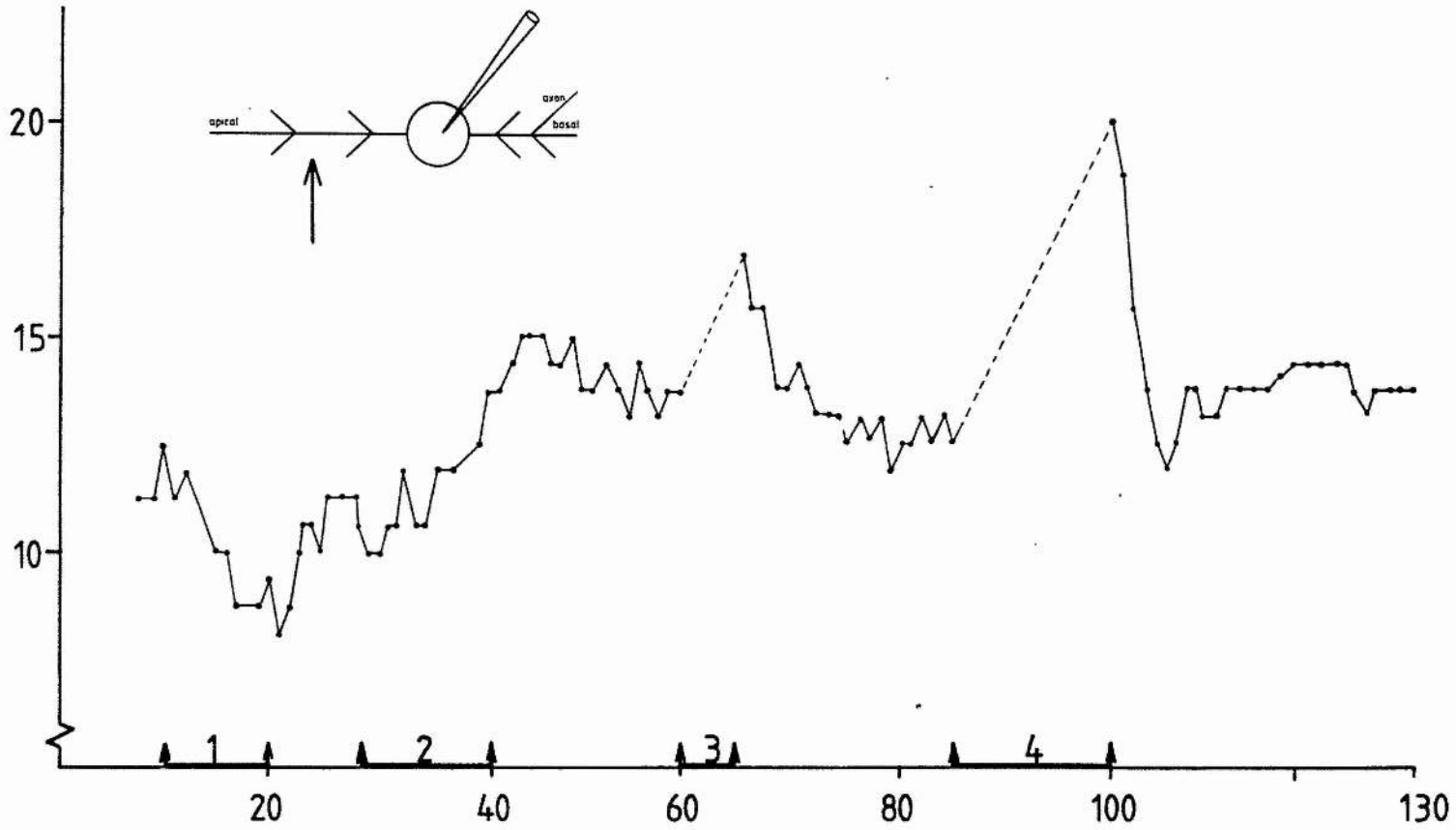

apical
axon
basal
20
15
10
1
2
3
4
20
40
60
80
100
130

FIGURE 3.9.

Size of depolarizing response of the dendrites near the cell body at different membrane potentials.

EDA iontophoresis (50nA).

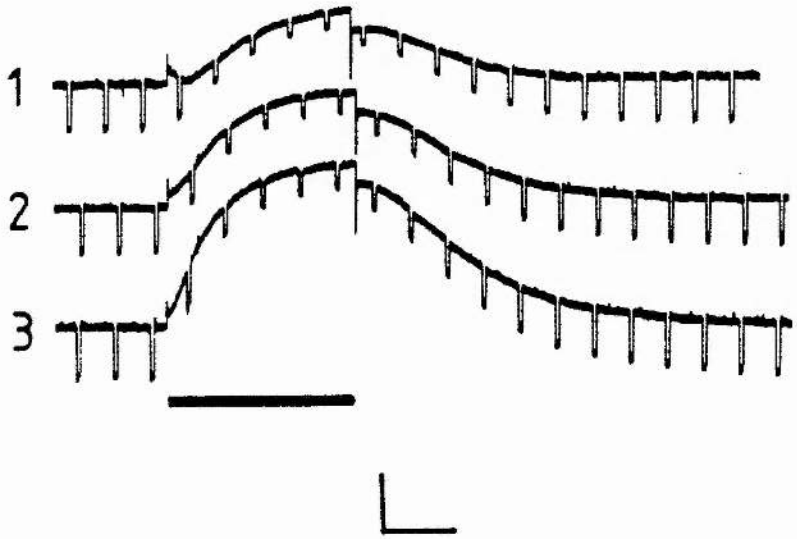
1. Cell body depolarized 10mV.
2. Cell body membrane potential.
3. Cell body hyperpolarized 10mV.

$V_m$ -65mV.

Scale bars 5mV, 2s.

Note that the responses become larger when the membrane is hyperpolarized.

Diagram shows position of iontophoresis.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3

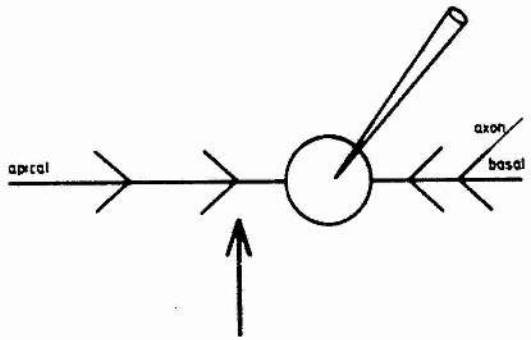

apical
axon
basal

FIGURE 3.10.

There is no effect of high $Mg^{++}$/low $Ca^{++}$ ACSF on the depolarizing response of the dendrites to EDA (40nA).

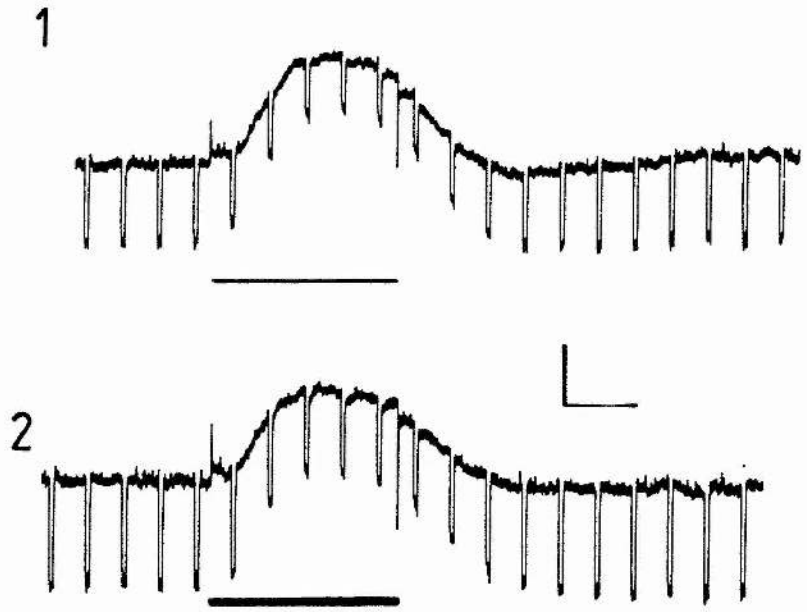
1. Control.
2. In the altered ACSF. (12 min after switching solution)

$V_m$ -65mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

Reduction in the conductance increase in 2. compared with 1. may be due to the block of the underlying hyperpolarizing response by the altered ACSF.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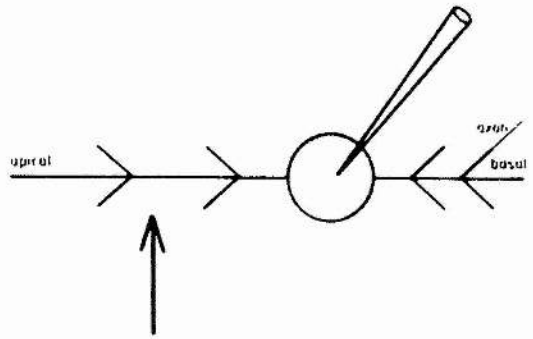
2


apical
basal

with ACSF containing 2mM cobalt chloride had no effect on the size of the depolarizing response of the dendrites, suggesting that the response was not calcium ion dependent.

3.3.1.2. HYPERPOLARIZING RESPONSES OF THE DENDRITES.

In addition to the depolarizing of the dendrites, EDA regularly produced a hyperpolarizing response of the dendrites too. This was accompanied by an increase in conductance and usually occurred after the depolarizing response, although it could be found on its own (FIG 3.32.A). The reversal potential of this response was about 15mV more negative than the membrane potential. On careful inspection of the depolarizing responses produced by GABA, it was sometimes also possible to detect a small hyperpolarization (FIG 3.4.3). Subsequently, it became clear that in order to obtain this response with GABA other than by luck, the iontophoretic electrode had to be placed in a specific, but unknown, location in the dendrites.

The iontophoretic transport numbers of GABA and EDA are similar (Perkins and Stone, 1982) allowing ejection currents to be compared as doses. As can be seen from many of the figures, GABA and EDA were roughly equipotent in eliciting the dendritic depolarization, but EDA was more effective at producing the dendritic hyperpolarization. As the dose response curves with GABA and EDA had the same maximum, it appears that GABA and EDA were equipotent.

Both the hyperpolarizing and depolarizing responses were inhibitory but, for the same shift of potential, the hyperpolarizing response was the most effective in reducing spiking. FIG 3.11 shows a depolarizing response of the dendrites to EDA followed by a hyperpolarizing response of the dendrites to EDA. The rate of firing was decreased by the depolarization, but was reduced to zero by the hyperpolarization.

### 3.3.2. RESPONSES OF THE CELL BODY.

Iontophoresis of GABA near the cell body produced a hyperpolarization (FIG 3.12.1). The response did not desensitize. The size of the response often changed during the course of an experiment even though the membrane potential was stable. As the size of the response was always influenced by changes in the membrane potential of the cell body, the reversal potential of the hyperpolarizing response of the cell body was estimated by passing current intracellularly. The reversal potential measured by current injection was between 0mV and 10mV more negative than the membrane potential. The reversal potential for 3 experiments is shown in FIG 3.13A. On both occasions that the method of Assaf et al (1981) was used, a reversal potential of 8mV more negative than the membrane potential was found. One example is shown in FIG 3.13.B. Small ejection currents were necessary because depolarizing responses appeared if the ejection current was too high.

FIGURE 3.11.

Respones produced by EDA and GABA are inhibitory.

1. EDA iontophoresis (60nA).

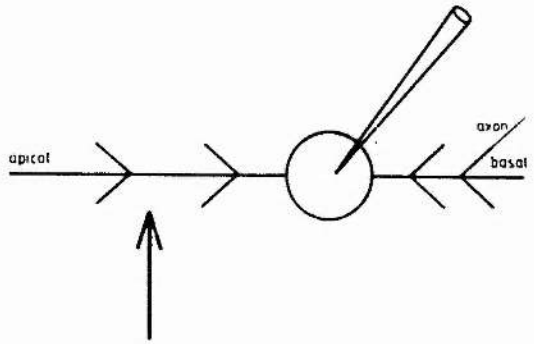
   $V_m$ -60mV.

2. GABA iontophoresis (50nA).

   $V_m$ -55mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.




1

2

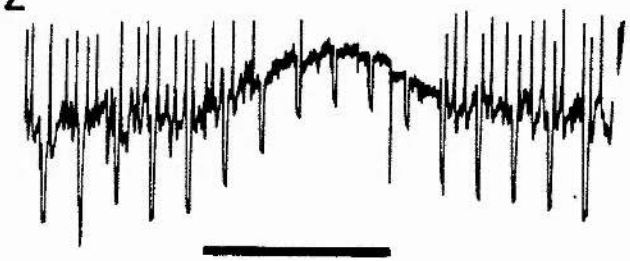


FIGURE 3.12.

Responses of the cell body to;

1. GABA (40nA),

   $V_m$ -55mV,

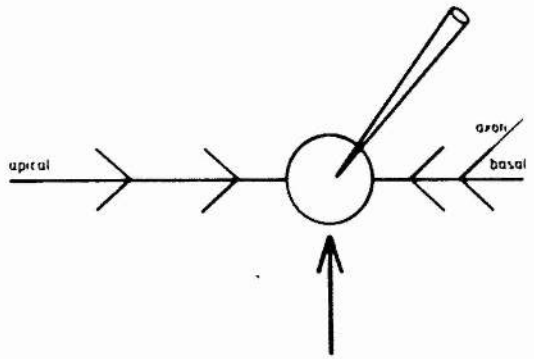
2. EDA (50nA),

   $V_m$ -60mV.

Scale bars 5mV, 2s.

Note differences in the shape of the responses.

Diagram shows the position of iontophoresis.

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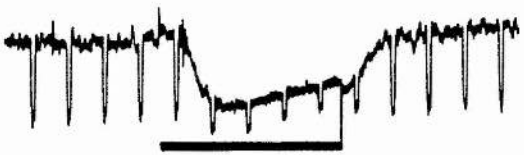


2

FIGURE 3.13.

Estimation of reversal potential by two methods.

A. By current injection.

Closed circles; GABA intophoresis.

$V_m$ -57mV.

Open circles; EDA iontophoresis.

$V_m$ -65mV.

Crosses; GABA iontophoresis.

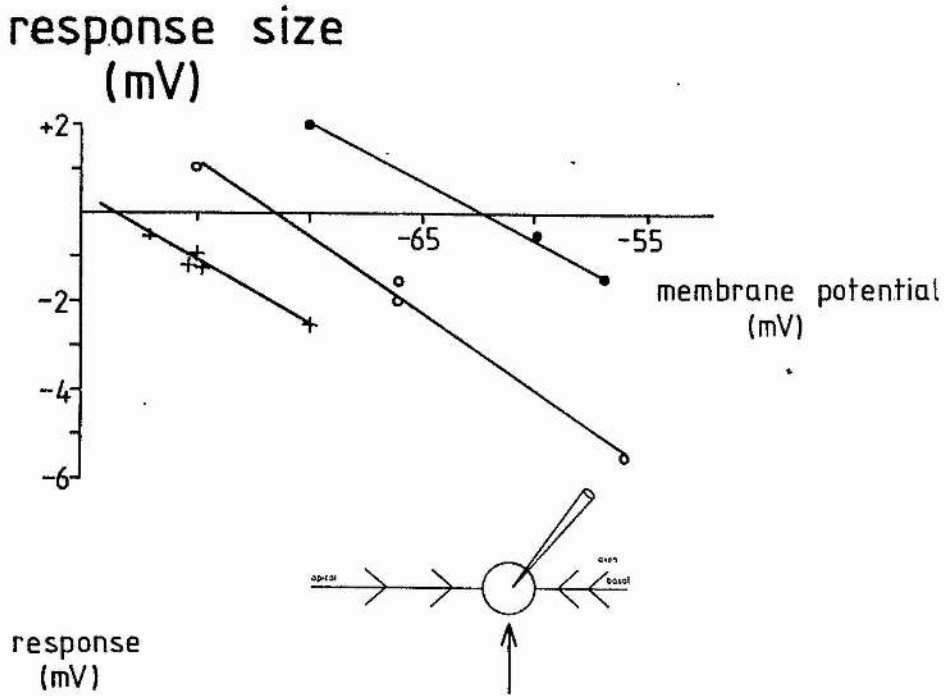
$V_m$ -75mV.

B. By the method of Assaf *et al*, (1981).

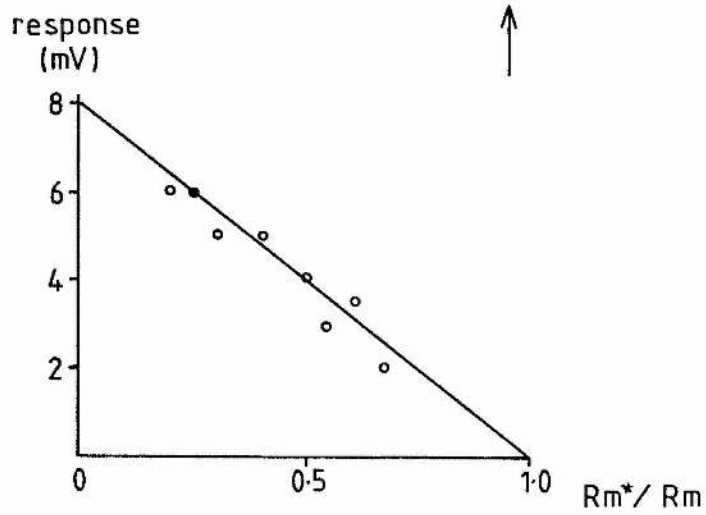
$V_m$ -70mV.

Diagram shoes the position of iontophoresis.

A


response size
(mV)
+2
-2
-4
-6
-65
-55
membrane potential
(mV)

B


response
(mV)
8
6
4
2
0
0·5
1·0
Rm*/ Rm

EDA also produced a hyperpolarizing response of the cell body (FIG 3.12.2). This response was shaped differently from the hyperpolarizing response to GABA. The reversal potential of this response, measured by displacing the membrane potential, was between 0mV and 10mV more negative than the membrane potential (FIG 3.13A). Typically, large iontophoretic artefacts were found when the iontophoretic electrode was near the cell body. The presence of these with a hyperpolarizing response suggested that the response was of the cell body and not of the dendrites.

A depolarizing response of the dendrites to EDA often underlay the hyperpolarizing response of the cell body to EDA. In FIG 3.14.2, a pure hyperpolarizing response is seen on the top border (membrane potential) of the envelope of hyperpolarizing current pulses. On the lower border, though, (ie at a hyperpolarized level) a depolarizing response to EDA is seen which does not coincide with the hyperpolarizing response on the top border. If this depolarization were to be increased in size (see section 3.5) then an overt depolarization would be seen. In comparison, a depolarizing response of the dendrites to EDA is shown in FIG 3.14.1. Here, both the response at the membrane potential and the response at the hyperpolarized level coincide, indicating that only one response is present.

To look at the hyperpolarization of the cell body alone,

FIGURE 3.14.

Envelope of different responses. Hyperpolarizing pulses of 450ms duration were passed into the cell. The upper border shows the response at the membrane potential, the lower border the response at the hyperpolarized level.

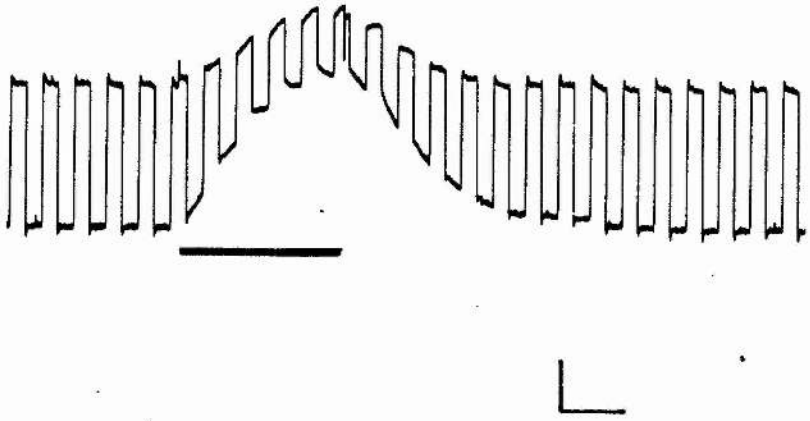
1. EDA iontophoresis (80nA) in the dendrites. Only depolarizations are seen.
2. EDA iontophoresis (60nA) on the cell body. The upper border shows only a hyperpolarization during the application.. The lower border shows a depolarization during the application.

$V_m$ -60mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

1



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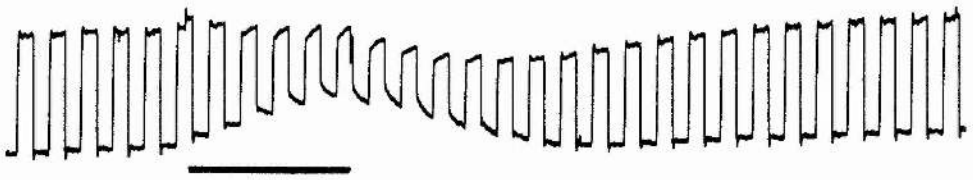
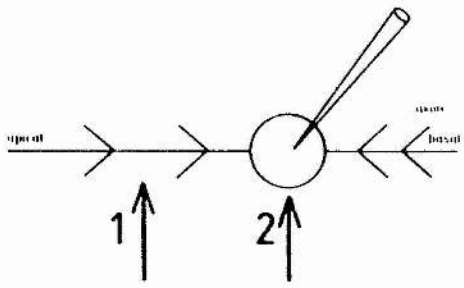




unaffected by the other responses, a different method of applying the drug was tried in two experiments in an effort to localize the application of drug. Instead of 5s applications at low iontophoretic currents, short pulses of 400nA (10ms to 100ms duration) were used. The log dose-response curve (LDRC) prepared by 5s applications at different iontophoretic currents was flat and with a low maximum. The crude data revealed interference by a depolarization in the hyperpolarizing response at the higher iontophoretic currents. The LDRC from the short pulse applications was steep and had a sigmoid shape. Although the size of the responses were less easy to measure because of their short duration, it was clear that they were not contaminated by other responses. The curves are shown in FIG 3.15.

FIGURE 3.15.

Comparison of LDRCs from two different methods of application of GABA to the cell body.

A. increasing ejection currents applied for 5s.

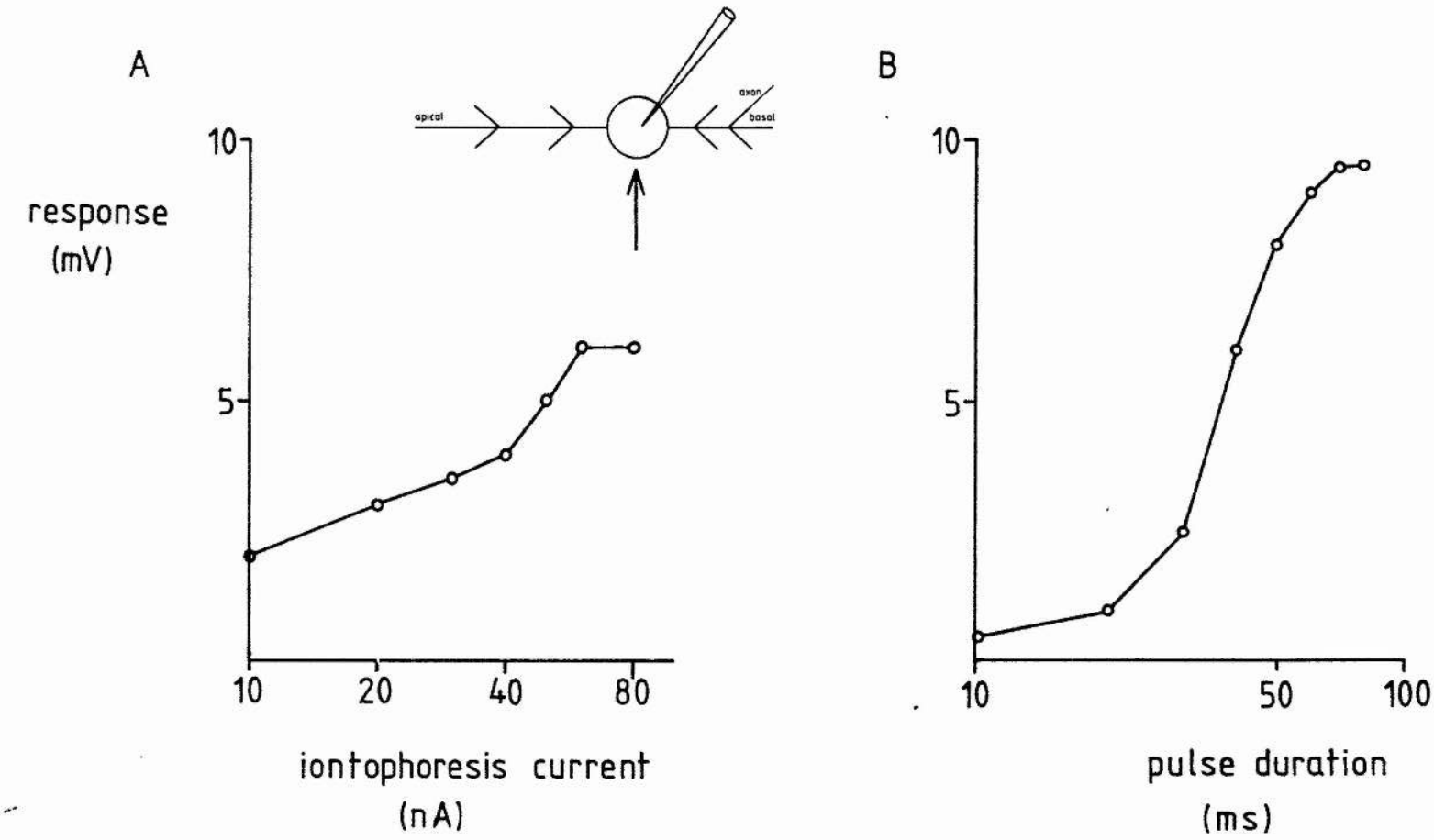
$V_m$ -70mV.

B. increasing duration of short pulses at 400nA.

$V_m$ -55mV.

Note sigmoid shape of the curve in B.

Diagram shows position of iontophoresis.


A
B
apical
axon
basal
10
5
response
(mV)
10
20
40
80
iontophoresis current
(nA)
10
50
100
pulse duration
(ms)

## 3.4 RESPONSES OF THE NEURONES TO BACLOFEN.

Iontophoresis of baclofen had no effect on either the dendrites or the cell body. Baclofen is poorly charged in solution and so may have carried very little of the iontophoretic current. Baclofen (10μM) was applied by bath application in two experiments. On both occasions baclofen produced a hyperpolarization of about 7mV in the course of a few minutes after application was started. The response was accompanied by a 14% increase in conductance. The membrane potential recovered on changing back to normal ACSF (FIG 3.16A).

In the presence of baclofen (10μM) in the bath, EDA only produced depolarizing responses of the dendrites without the hyperpolarizing responses which were seen in the abscence of baclofen (FIG 3.14.B).

The lack of a hyperpolarizing response to EDA can be explained as an action of baclofen interfering with the action of EDA which produces the hyperpolarization. There are, however, other ways to explain the data:

1. That baclofen had hyperpolarized the neurone to such a degree that the hyperpolarization produced by EDA was not seen (it might have been possible to depolarize the neurone to the original membrane potential).

2. The EDA hyperpolarization (small anyway) was lost

FIGURE 3.16.

Effect of baclofen (10μM) on the membrane potential and the sensitivity of the dendrites to the iontophoresis of EDA (50nA).

A. Response of the neurone to baclofen and the recovery later.

Bar shows the time between the switching of the tap that controlled the solutions.

$V_m$ -55mV.

Scale bars 5mV, 10s.

B. Responses of the dendrites to EDA.

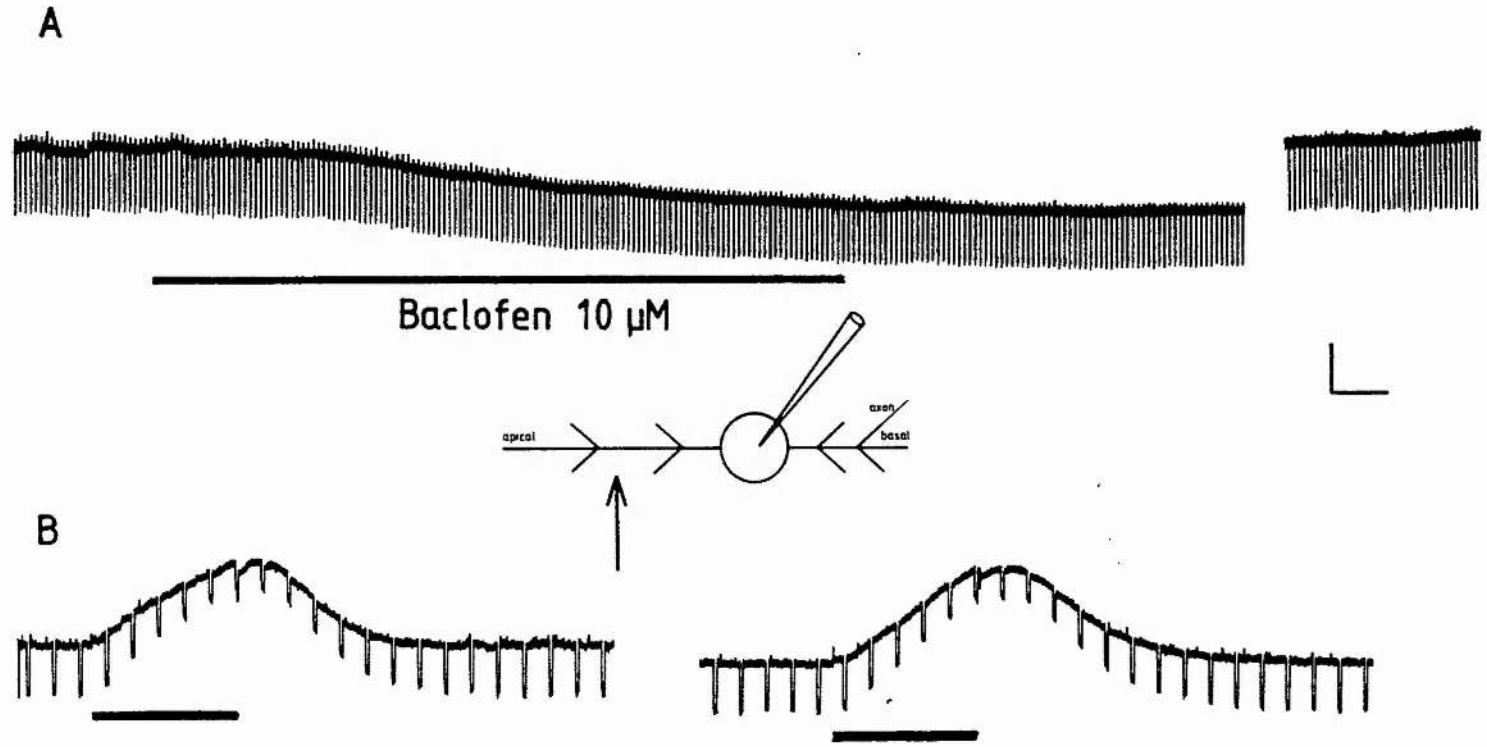
1. control.

2. In the presence of baclofen (10μM). First response obtained 5 min after switching the tap that controlled the solution.

$V_m$ -55mV.

Scale bars 5mv, 2s.

Diagram shows the position of iontophoresis.




1. 2.

because of electrode movement.

3. That baclofen had caused the release of GABA. This GABA was then responsible for the hyperpolarization of the neurone.

## 3.5 ION SUBSTITUTION EXPERIMENTS.

### 3.5.1. DEPOLARIZING RESPONSES OF THE DENDRITES TO GABA AND EDA.

The size of the depolarizing response to GABA or EDA was increased 2-4 fold in 6mM $Cl^-$ and 1.5-2 fold 88mM $Cl^-$ (FIG 3.17). The change in ground potential in FIG 3.17 (arrowed) shows that the new solution had reached the electrode in the bath in 30s. Low $Cl^-$ (isethionate substituted, 88mM $Cl^-$) ACSF had little effect on the I/V curve of the neurone (FIG 3.18) and no effect on the ground potential (changes in which may have disguised changes in membrane potential). The low $Cl^-$ ACSF (6mM), as shown in FIG 3.17B, produced a 30mV change in ground potential (arrowed). The depolarizing response of the dendrites to GABA and EDA was the only response of these neurones consistently affected (28 neurones out of 28) by low $Cl^-$ ACSF.

In two neurones where tests were made, low $Cl^-$ ACSF (88mM $Cl^-$) affected the desensitization of the depolarizing response of the dendrites to GABA. In normal ACSF, there appeared to be little desensitization produced by the high iontophoretic current used in the preparation of the the DRC. In low $Cl^-$ ACSF (88mM $Cl^-$), the high iontophoretic currents reduced the size of the depolarizing responses produced by subsequent applications of GABA. Four LDRCs are shown in FIG 3.19. The two right hand curves were prepared in normal ACSF.

FIGURE 3.17.

Effect of low $Cl^-$ ACSFs on the depolarizing response of the dendrites.

A. EDA iontophoresis (50nA).

1; control.

2; in low $Cl^-$ ACSF (88mM $Cl^-$), 7 min after switching the tap that controlled the solution.

3; wash.

Scale bar 5mV, 2s.

$V_m$ -65mV.

B.GABA iontophoresis (40nA).

1. Control response.

2. Response in 6mM $Cl^-$ ACSF, 5 min after switching the tap that controlled the solutions.

Change in ground potential arrowed.

3. Wash.

$V_m$ -65mV.

Scale bars 5mV, 10s.

Diagram shows the position of iontophoresis.

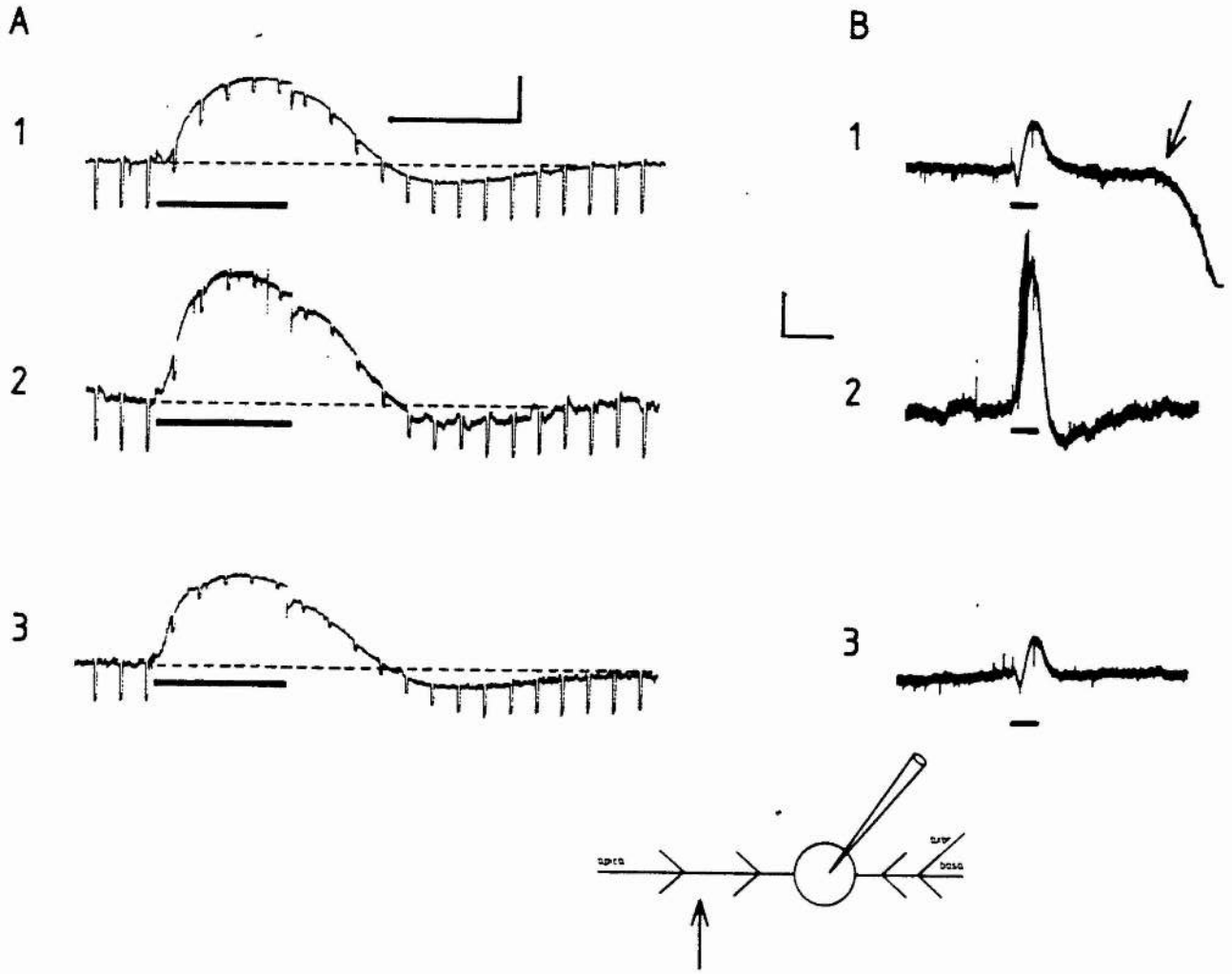

A
1
2
3
B
1
2
3

FIGURE 3.18.

Current / voltage relationship in normal ACSF and low $Cl^-$ ACSF (88mM $Cl^-$), starting 5 min after switching the tap that controlled the solutions.

Closed circles; normal ACSF.

Open circles; low $Cl^-$ ACSF.

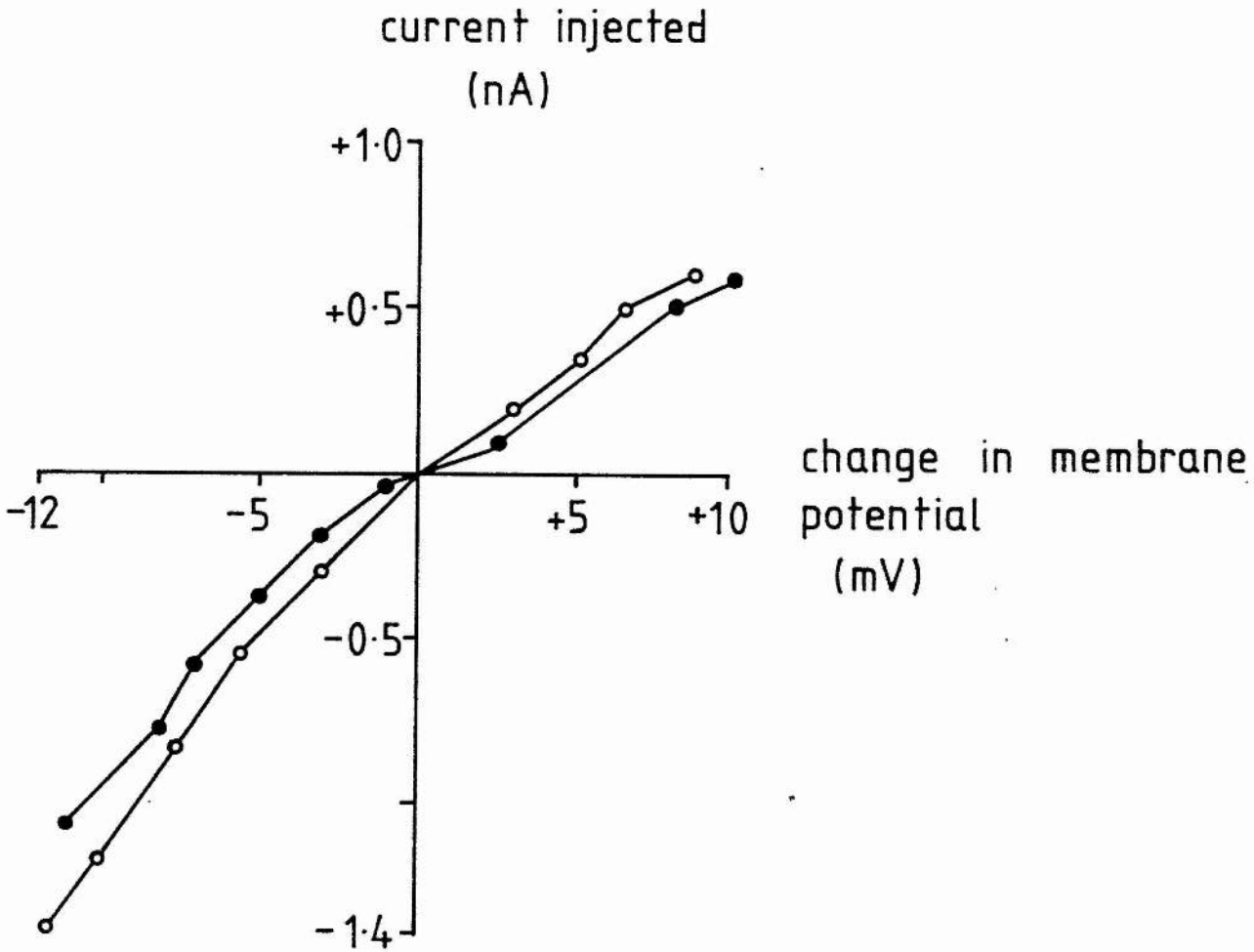

current injected
(nA)
+1·0
+0·5
-0·5
-1·4
-12
-5
+5
+10
change in membrane
potential
(mV)

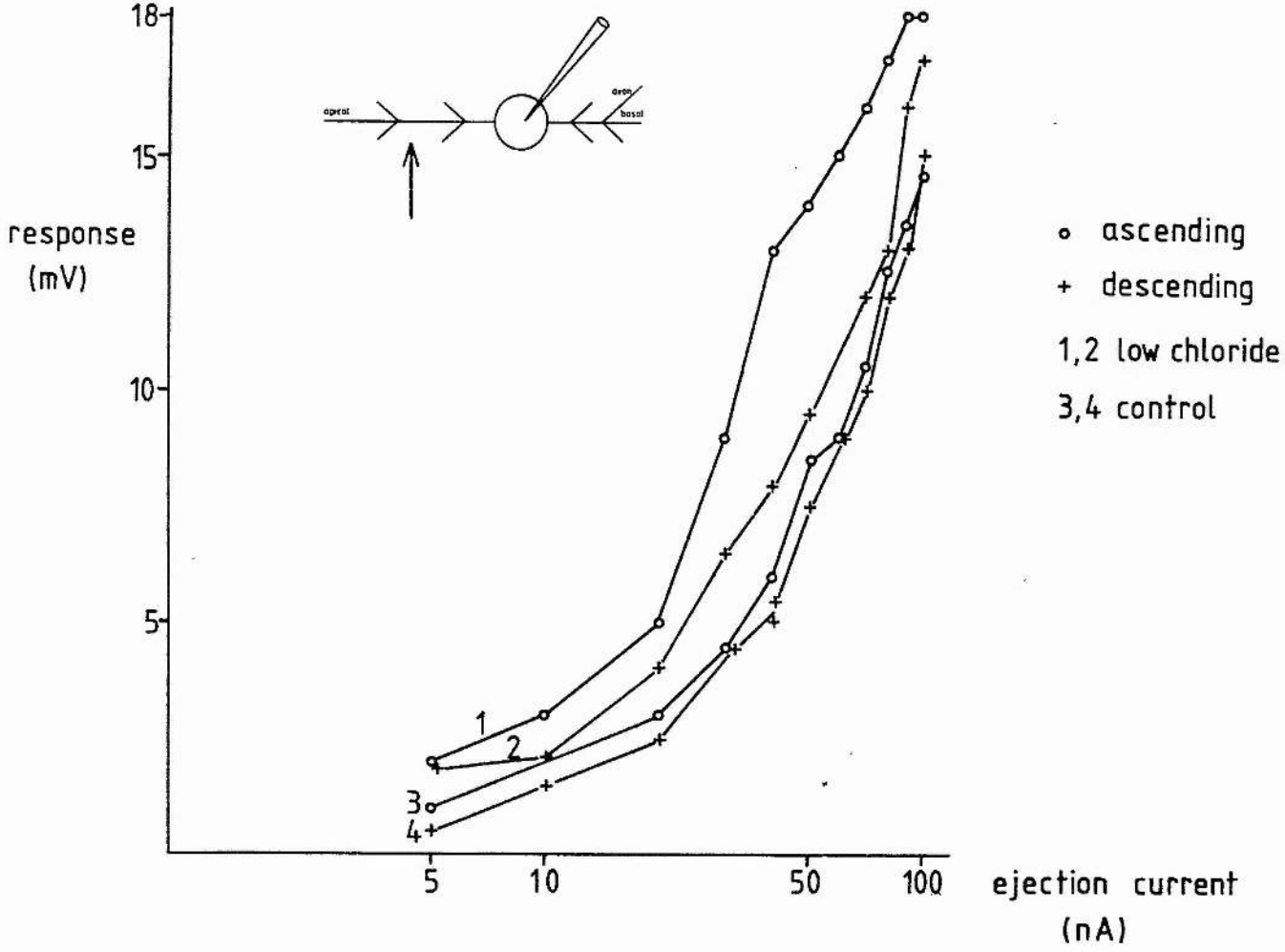
FIGURE 3.19.

Desenstization of the depolarizing response of the dendrites to GABA during the preparation of dose response curves. ´Ascending´ means the application of GABA at increasing ejection currents each minute; ´descending´ means the application of GABA at decreasing ejection currents each minute.

$V_m$ -68mV.

Note the separation of the ascending and descending curves in low $Cl^-$ ACSF.

The preparation of the curves was started 6 min after switching the tap that controlled the solutions.


18
15
10
5
response
(mV)
apical
basal
1
2
3
4
5
10
50
100
ejection current
(nA)
ascending
descending
1,2 low chloride
3,4 control

by ascending from 5 to 100nA ejection current and then descending. There was a little rightward shift of the descending curve produced by the high iontophoretic currents of the ascending LDRC. The two left hand curves were prepared in low $Cl^-$ ACSF. The descending curve was then quite separate from the ascending curve. From the chart record, desensitization was clearly occurring. An anomaly of iontophoresis was not responsible for the observation. Experiments involving the substitution of $Cl^-$, however, do not rule out the involvement of another ion in the depolarizing response of the dendrites. There are two ways of finding out whether more than one ion is involved.

1. The effect of substituting other ions can be examined.

2. The change in the reversal potential of the depolarizing response when perfused with low $Cl^-$ ACSF can be quantified.

The effect of low $Na^+$ ACSF on the depolarizing response of the dendrites to EDA was clear, although the explanation may be complicated. The size of the response was reversibly reduced when the $[Na^+]_o$ was reduced to 26mM with choline as the substituent cation (n=4)(FIG 3.20A.). There was no direct effect of choline on the neurone. The results with GABA were quite different. The size of the depolarizing response of the dendrites to GABA increased when the $[Na^+]_o$ was reduced to 26mM with choline substituted (n=4)(FIG 3.20.B).

FIGURE 3.20.
Effect of different low $Na^+$ ACSFs on the dendritic responses to GABA and EDA.

A. EDA iontophoresis (50nA).
   1. control.
   2. in low $Na^+$ (26mM $Na^+$) ACSF, choline substituted, 7 min after switching the tap that controlled the solutions.
   3. wash.

   Scale bar 5mV, 2s.
   $V_m$ -65mV.

B. GABA iontophoresis (20nA).
   1. control.
   2. in low $Na^+$ ACSF (88mM $Na^+$), choline substituted, 7 min after switching the tap that controlled the solutions.

   Scale bar 5mV, 10s.
   $V_m$ -70mV.

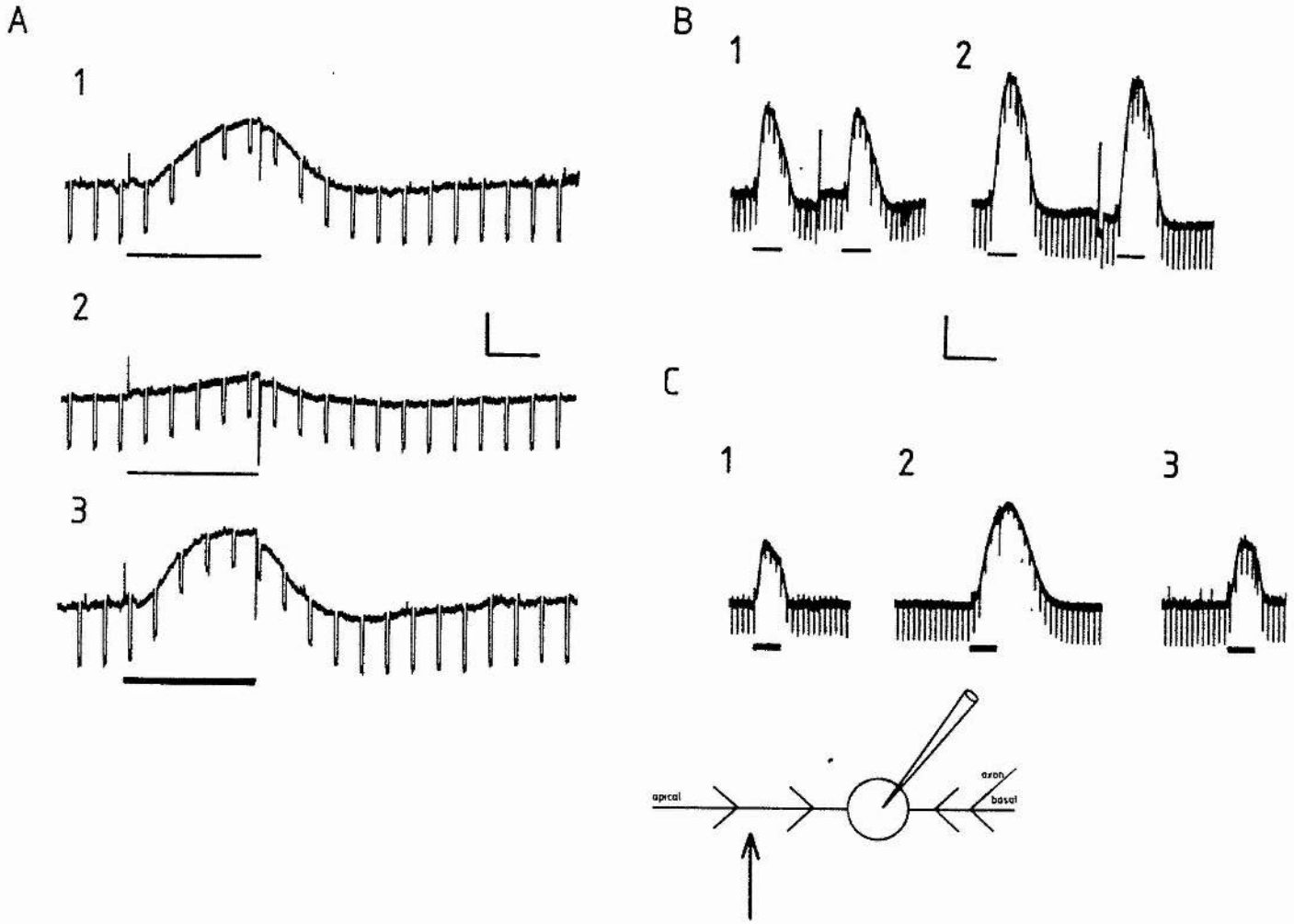
C. GABA iontophoresis (20nA).
   1. control.

   Scale bar 5mV, 10s.
   2. in low $Na^+$, low $Cl^-$ ACSF, (26mM $Na^+$, 6mM $Cl^-$), sucrose substituted, 4 min after switching the tap that controlled the solutions.

   Scale bar 10mV, 10s.
   3. wash.

   Scale bar 5mV, 10s.
   $V_m$ -60mV.

Note the opposite effects of low $Na^+$ ACSF on the responses to GABA or EDA.
Diagram shows the position of iontophoresis.


A
1
2
3
B
1
2
C
1
2
3
apical
axon
basal

When NaCl was substituted isotonically with sucrose, the depolarizing response of the dendrites to GABA increased in size also (n=3)(FIG 3.20.C). This was at least partly the result of the reduction in $[Cl^-]_o$. The response was also prolonged. Removing all the $Na^+$ from the solution and substituting with choline had undesirable consequences; usually the membrane potential changed, complicating the measurement of responses, and the input resistance increased, presumably as a result of a decrease in the $Na^+$ leakage conductance.

The depolarizing response of the dendrites to GABA or EDA was unaffected by a six-fold reduction in $K^+$. Perfusion of the slice with low $K^+$ ACSF (0.63mM $K^+$) hyperpolarized the neurone by between 1mV and 12mV. This sometimes produced a small increase in the size of the depolarizing response.

As the results of $Na^+$ substitution were difficult to interpret and could not answer the question of the involvement of $Na^+$ in the depolarizing response of the dendrites, the second approach outlined above was used. If the depolarizing response is entirely dependent on $Cl^-$ then changes in $[Cl^-]_o$ should change the reversal potential (ie $E_{Cl}$) by an amount predictable from the Nernst equation for $Cl^-$, assuming the membrane behaves as a linear $Cl^-$ electrode. In practice, it proved difficult to measure the changes in reversal potential. Intracellular current injections, although

sometimes successful in changing the size of the depolarizing responses in the dendrites, had an unknown effect on the actual membrane potential of the dendrites. On rare occasions, a depolarizing response of the dendrites was found near the cell body. Then, the reversal potential could be measured by displacing the membrane potential of the cell body. FIG 3.21 shows the reversal potential, measured by current injection, of a depolarizing response found very close to the cell body. It was assumed that the dendritic membrane potential was the same as the potential measured by the electrode. A plot of membrane potential vs. response amplitude was drawn which gives the reversal potential (response amplitude zero) at -66.8mV with the membrane potential of -75mV. In low $Cl^-$ ACSF (88mM $Cl^-$, $E_{Cl}$ = 10mV), the reversal potential changed 5mV to -61.8mV, rather less than the theoretical change for a purely $Cl^-$-dependent response of 10mV, This suggests that the response was not entirely $Cl^-$-dependent.

The more complicated treatment of Assaf et al (1981) (described fully in Methods) was used to measure the reversal potential of responses remote from current injection. Results were taken from 13 experiments. Generally, the reversal potential of the depolarizing response of the dendrites in normal ACSF was between 10mV and 25mV less negative than the membrane potential. When the $[Cl^-]_o$ was reduced to 88mM, producing a change in $E_{Cl}$ of 10mV less negative than $E_{Cl}$ in normal ACSF, the reversal potential of the depolarizing

FIGURE 3.21.

Reversal potential of depolarizing response of the dendrites to GABA obtained near the cell body.

GABA iontophoresis (30nA).

Control response; inversion potential was -66.8mV.

Response in low $Cl^-$ ACSF (88mM $Cl^-$); inversion potential was -61.8mV,

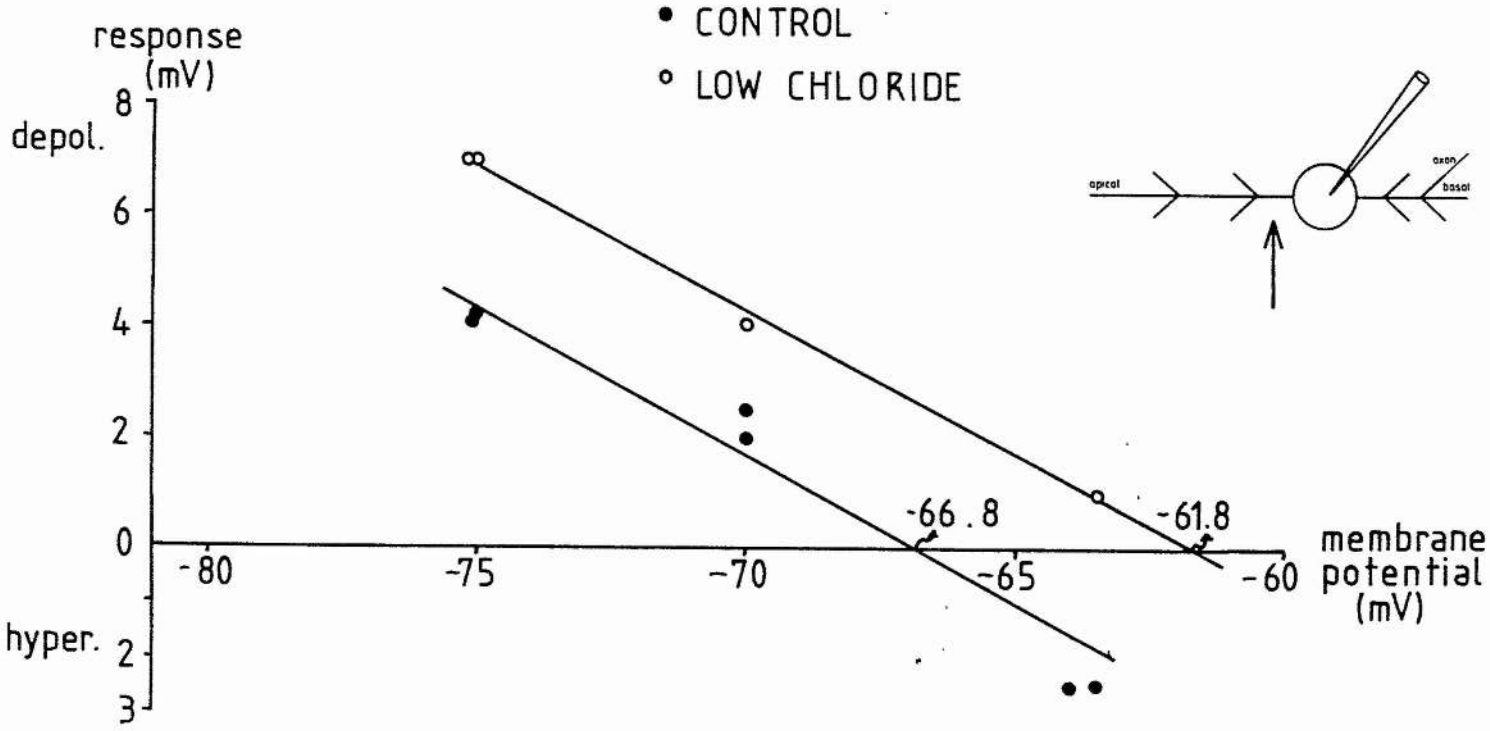
starting 6 min after switching the tap that controlled the solutions.

Change in inversion potential was 5mV.

$V_m$ -75mV.

Change in $E_{Cl}$ was 10mV.

Diagram shows the position of iontophoresis.


response
(mV)
depol.
hyper.
8
6
4
2
0
2
3
-80
-75
-70
-65
-60
-66.8
-61.8
membrane
potential
(mV)
CONTROL
LOW CHLORIDE
apical
basal
axon

response changed to between 5mV and 11mV less negative than the reversal potential in normal ACSF. Although there are problems of interpretation of these results (see Discussion), it appears that the depolarizing response of the dendrites to GABA or EDA was not solely dependent on $Cl^-$. The reversal potentials and shifts of reversal potential are given in TABLE 3. FIG 3.22 shows how the data were plotted to give the reversal potentials and how low $Cl^-$ ACSF (88mM $Cl^-$) changed the reversal potential in two experiments, one with a small change in $E_{rev}$ and one with a change in $E_{rev}$ near to the theoretical value of 10mV. The straight lines were calculated by linear regression analysis.

### 3.5.2. HYPERPOLARIZING RESPONSE OF THE DENDRITES.

This response, common with EDA but rare with GABA (10 out of 270 experiments) was unaffected by low $Cl^-$ ACSF, as shown in FIG 3.16A. The shape of the hyperpolarizing response was different in low $Na^+$ ACSF, becoming smaller and more prolonged. The size of the hyperpolarizing response of the dendrites to EDA was increased in low $K^+$ ACSF (0.63mM $K^+$) in 3 out of 5 experiments (FIG 3.23). The lack of effect of the low $K^+$ ACSF on the hyperpolarizing response in one of the the other two experiments is not a negative result, since the size of the response remained constant despite a hyperpolarization of the membrane potential of 12mV. These results suggest that the dendritic hyperpolarizing response to EDA was $K^+$ dependent, but not $Na^+$- or $Cl^-$- dependent.

FIGURE 3.22.

Reversal potentials of the depolarizing response of the dendrites to GABA. Measured by the method of Assaf et al (1981). Plot of response size against conductance ratio.

A. control line y-intercept = 16.8mV.

low chloride line y-intercept = 21.6mV.

Change in $E_{rev}$ = 4.8mV.

$V_m$ -60mV.

B. control line y-intercept = 20.9mV.

low chloride line y-intercept = 29.9mV.

Change in $E_{rev}$ = 9mV.

$V_m$ -65mV. The change in $E_{rev}$ is not the same as the change in $E_{Cl}$ (10mV change).

Diagram shows the position of iontophoresis.

Note that regression lines do not pass through 1.0. This may be due to the presence of an underlying response, although none was detected. An underlying response to GABA, accompanied by an increase in conductance would reduce the conductance ratio even when the overall response to GABA was depolarizing.

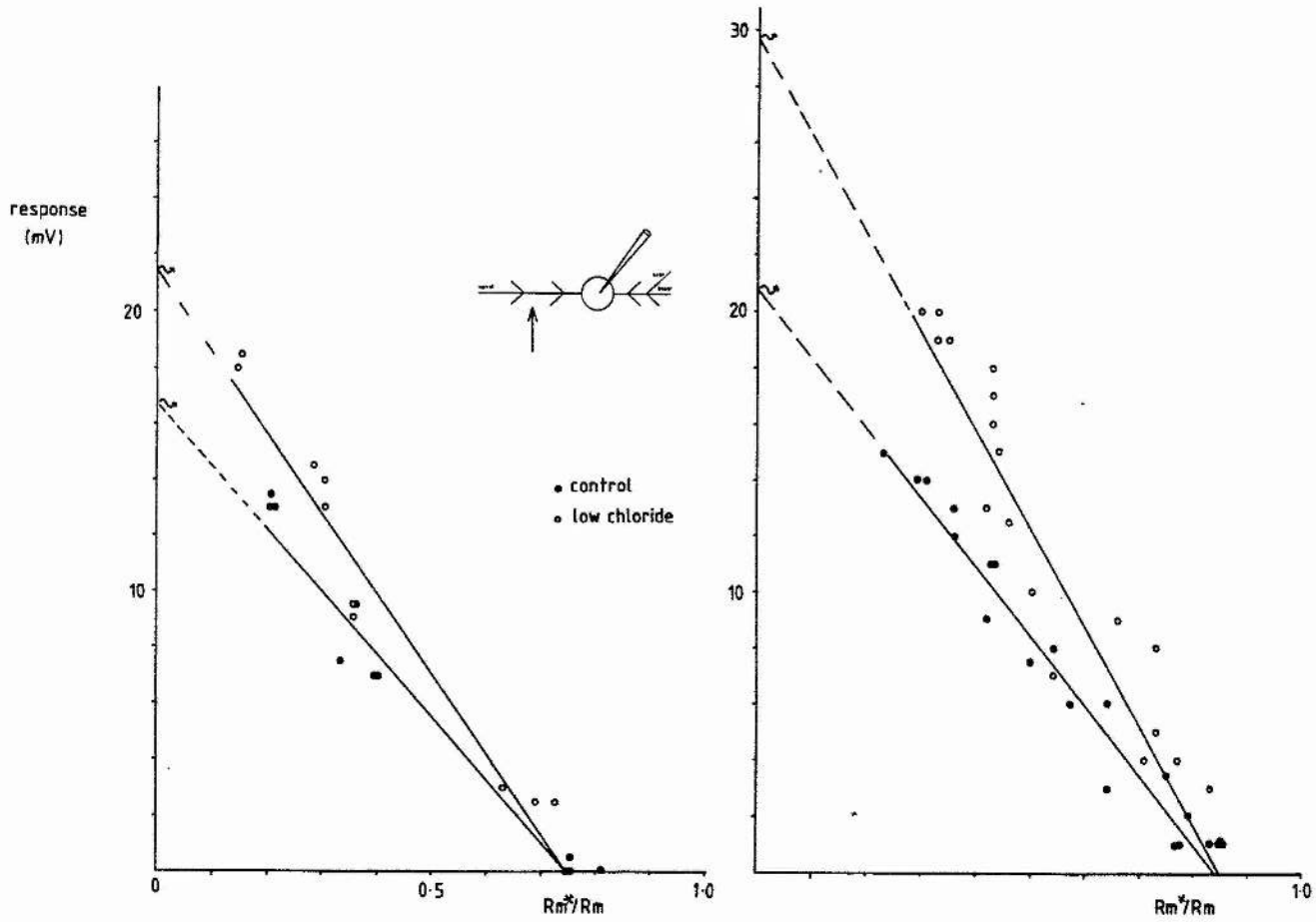

response
(mV)
20
10
0
0·5
1·0
Rm*/Rm
control
low chloride
30
20
10
1·0
Rm*/Rm

FIGURE 3.23.

Effect of low $K^+$ ACSF (0.63mM $K^+$) on a biphasic response of the dendrites to EDA (60nA).

1. Control response.
2. Response in low $K^+$ ACSF 11 min after switching the tap that controlled the solutions.
3. Wash.

$V_m$ -60mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

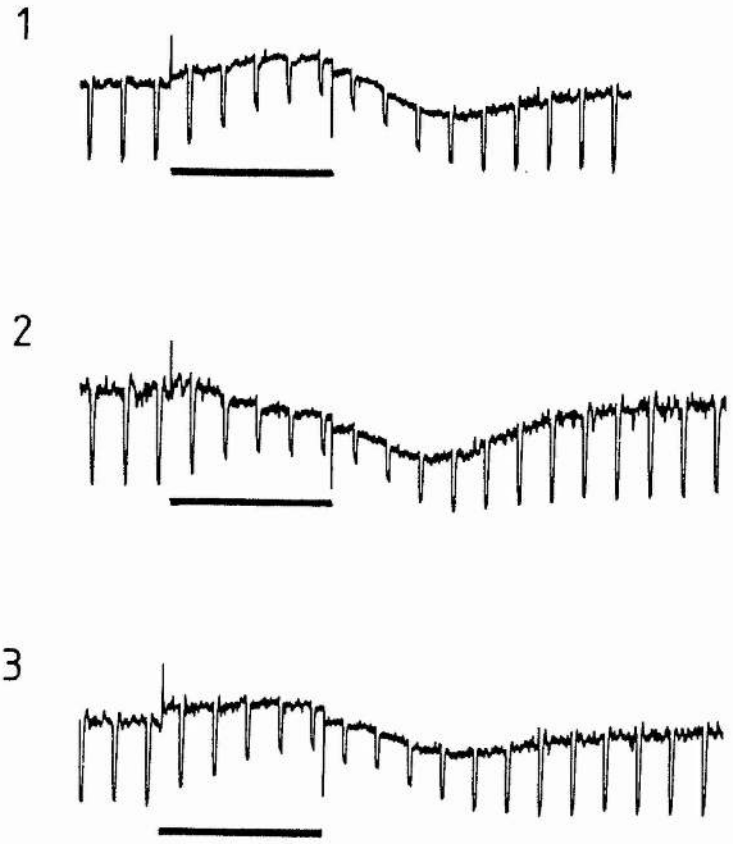

1
2
3
apical
axon
basal

TABLE 3.

Reversal potential of the depolarizing response of the dendrites to GABA or EDA for all the neurones which it was measured. Changes in the reversal potential produced by low $Cl^-$ ACSF (88mM $Cl^-$) are also shown. G; GABA. $-E_{rev}$ C; control reversal potential $-E_{rev}$ LC; reversal potential in low $Cl^-$ ACSF.

| TRACE | AGONIST | $-V_m$ | $-E_{rev}C$ | $-E_{rev}$ LC | $\Delta E_{rev}$ |
|---|---|---|---|---|---|
| 116 | GABA | 55 | 40 | | |
| 123 | GABA | 55 | 41 | | |
| 129 | EDA | 55 | 42 | | |
| 137 | EDA | 60 | 50 | | |
| 142 | EDA | 55 | 38 | | |
| 147 | GABA | 62 | 53 | | |
| 113 | GABA | 70 | 57 | 48 | 9 |
| 148 | GABA | 55 | 43 | 36 | 7 |
| 169 | GABA | 65 | 44 | 35 | 9 |
| 170 | GABA | 68 | 49 | 37 | 11 |
| 182 | GABA | 60 | 51 | 41 | 10 |
| 185 | GABA | 60 | 42 | 32 | 10 |
| 187 | GABA | 70 | 54 | 49 | 5 |
| 191 | GABA | 73 | 56 | 51 | 5 |
| 203 | GABA | 75 | 66 | 61 | 5 |

### 3.5.3.HYPERPOLARIZING RESPONSE OF THE CELL BODY TO GABA AND EDA.

The hyperpolarization of the cell body was unaffected by low $Cl^-$ ACSF. The interpretation was complicated by the interference of the depolarizing responses of the dendrites. In some experiments, low $Cl^-$ ACSF apparently had an effect on the size of the response, but this can be explained by the increase in size of the contaminating depolarizing response of the dendrites. Such a result is shown in FIG 3.24.A. On close inspection of this figure, a small depolarizing response is seen before the hyperpolarizing response (FIG 3.24.A.1; depolarization arrowed), presumably because the EDA had diffused to responsive dendrites which depolarized. The whole response did not invert in low $Cl^-$ ACSF (88mM $Cl^-$). A tiny portion remained hyperpolarizing (FIG 3.24.A.2, arrowed). On washing (partial washout shown in FIG 3.24.A.3) there were clearly two phases to the response. A similar observation was made in another experiment (FIG 3.24.B) where, using GABA as the agonist, a pure hyperpolarizing response of the cell body was obtained (FIG 3.24.B.1.). In low $Cl^-$ (88mM $Cl^-$) a depolarization is clearly superimposed on the hyperpolarization (FIG 3.24.B.2.).

To examine the effect of low $Cl^-$ ACSF more closely, a depolarizing response to GABA with a small hyperpolarizing response preceding it was found near the cell body. The

FIGURE 3.24.

Effect of low $Cl^-$ ACSF (88mM $Cl^-$) on the response of the cell body to EDA and GABA.

A. EDA iontophoresis (50nA).

1. Control. Arrow shows small depolarizing response.

2. Response in low $Cl^-$ ACSF, 5 min after switching the tap that controlled the solutions. Arrow shows small hyperpolarizing response.

3. Partial wash, 6 min after switching the tap that controlled the solutions.

$V_m$ -55mV.

B. GABA iontophoresis (15nA).

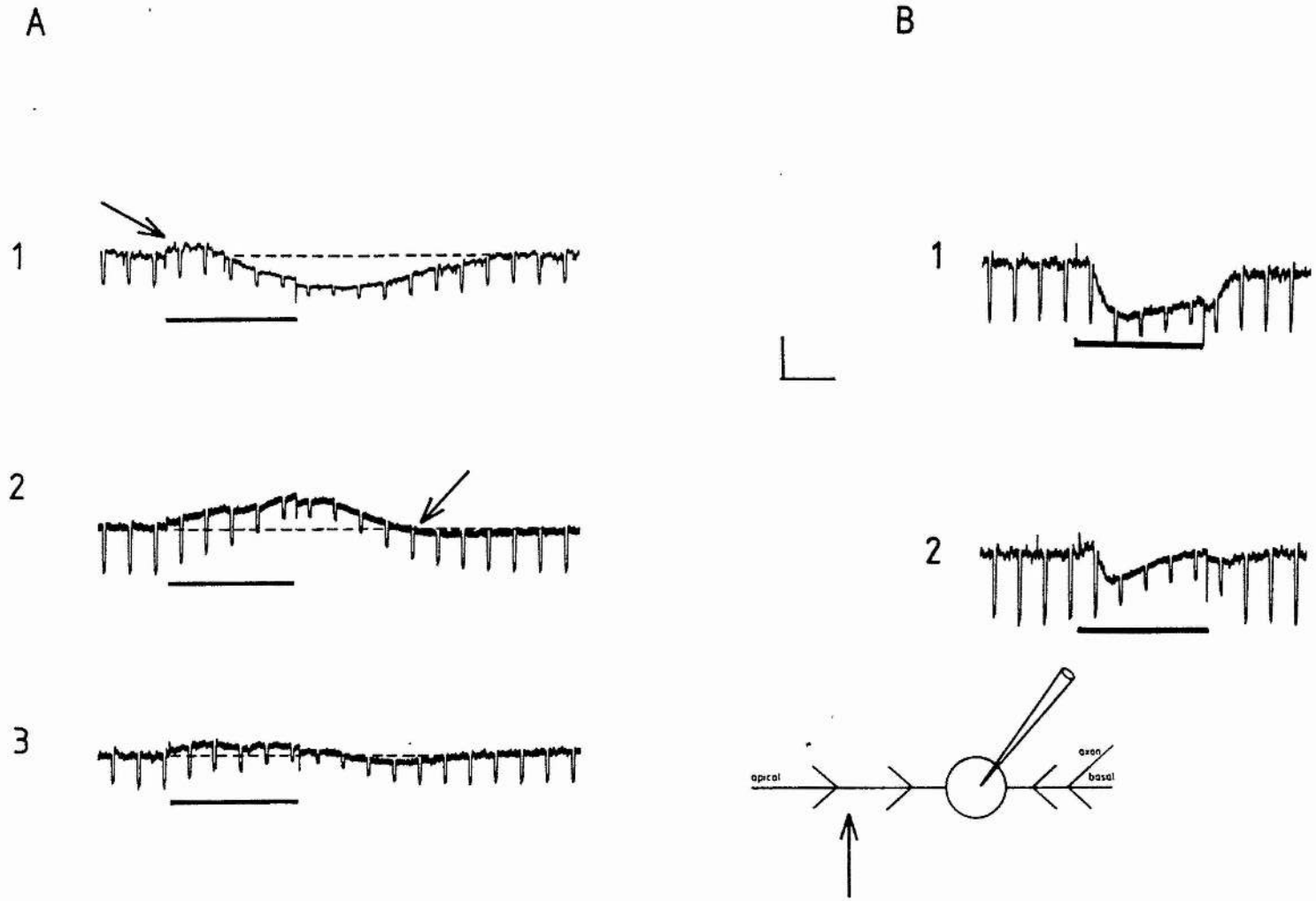
1. Control.

2. Response in low $Cl^-$ ACSF, 6 min after switching the tap that controlled the solutions.

$V_m$ -55mV.

Scale bars 5mV, 2s.

Diagram shows position of iontophoresis.


A
1
2
3
B
1
2
apical
axon
basal

neurone was depolarized by intracellular current injection by 5mV, 10mV and 15mV, and GABA applied at each potential (FIG 3.25.A). The hyperpolarizing response became more predominant as the neurone was depolarized. The same series was repeated in low $Cl^-$ ACSF (88mM $Cl^-$, FIG 3.25.B). The size of the hyperpolarization remained the same but the depolarization became larger. The series was repeated in normal ACSF (FIG 3.25.C). A full recovery was seen. It appears that the hyperpolarizing response to GABA was not chloride dependent.

The hyperpolarizing response of the cell body to GABA and EDA was increased in low $K^+$ ACSF (0.63mM $K^+$, FIG 3.26). Ten out of 13 neurones showed this effect. The results were complicated by the difficulty of finding a pure hyperpolarization without an accompanying depolarization.

### 3.5.4. SUMMARY OF ION SUBSTITUTION RESULTS.

The depolarizing response of the dendrites to GABA and EDA was $Cl^-$-dependent. The effect of $Na^+$ substitution was complicated. The dendritic hyperpolarizing response to EDA was $K^+$ dependent. GABA produced a hyperpolarizing response of the dendrites rarely. The hyperpolarizing response of the cell body to GABA or EDA, which could be distinguished from the hyperpolarizing response of the dendrites to GABA or EDA, was also $K^+$ dependent.

FIGURE 3.25.

Effect of low $Cl^-$ ACSF (88mM $Cl^-$) on a biphasic response obtained near the cell body.

GABA iontophoresis (45nA).

| A. Control responses. | B. In low $Cl^-$ ACSF. | C. Wash. |
|---|---|---|
| 1. 15mV depolarized. | 1. 15mV depolarized. | 1. 5mV depolarized. |
| 2. 10mV depolarized. | 2. 10mV depolarized. | 2. 10mV depolarized. |
| 3. 5mV depolarized. | 3. 5mV depolarized. | 3. 5mV depolarized. |

$V_m$ -55mV.

Scale bars 5mV, 2s. Responses in B. were obtained starting 4 min after switching the tap that controlled the solutions.

Note the lack of effect of low $Cl^-$ ACSF on the size of the hyperpolarizing response.

Diagram shows the position of iontophoresis.

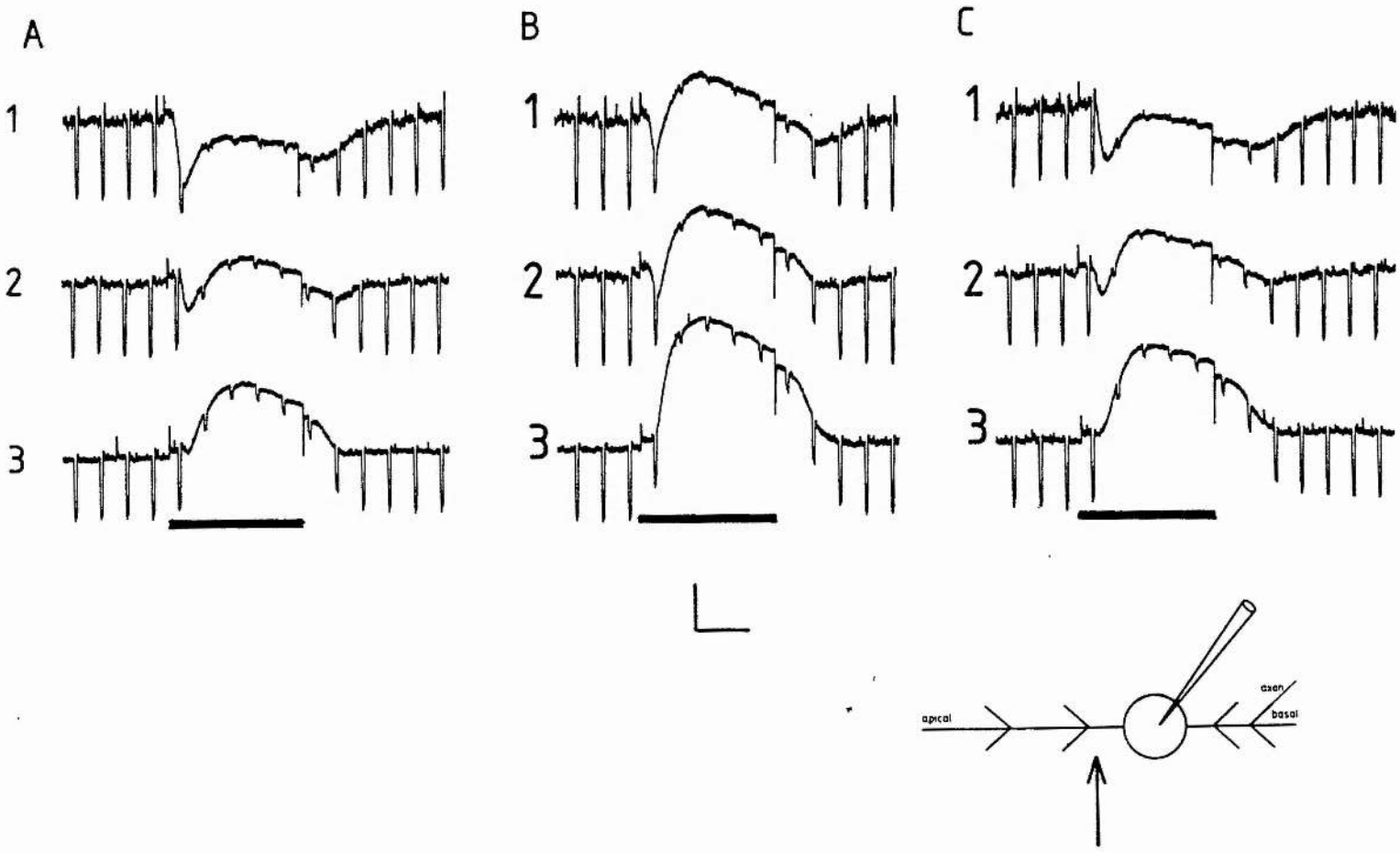

A
B
C
1
2
3
1
2
3
1
2
3
apical
axon
basal

FIGURE 3.26.

Effect of low $K^+$ ACSF (0.63mM $K^+$) on a biphasic response of the cell body.

GABA iontophoresis (20nA).

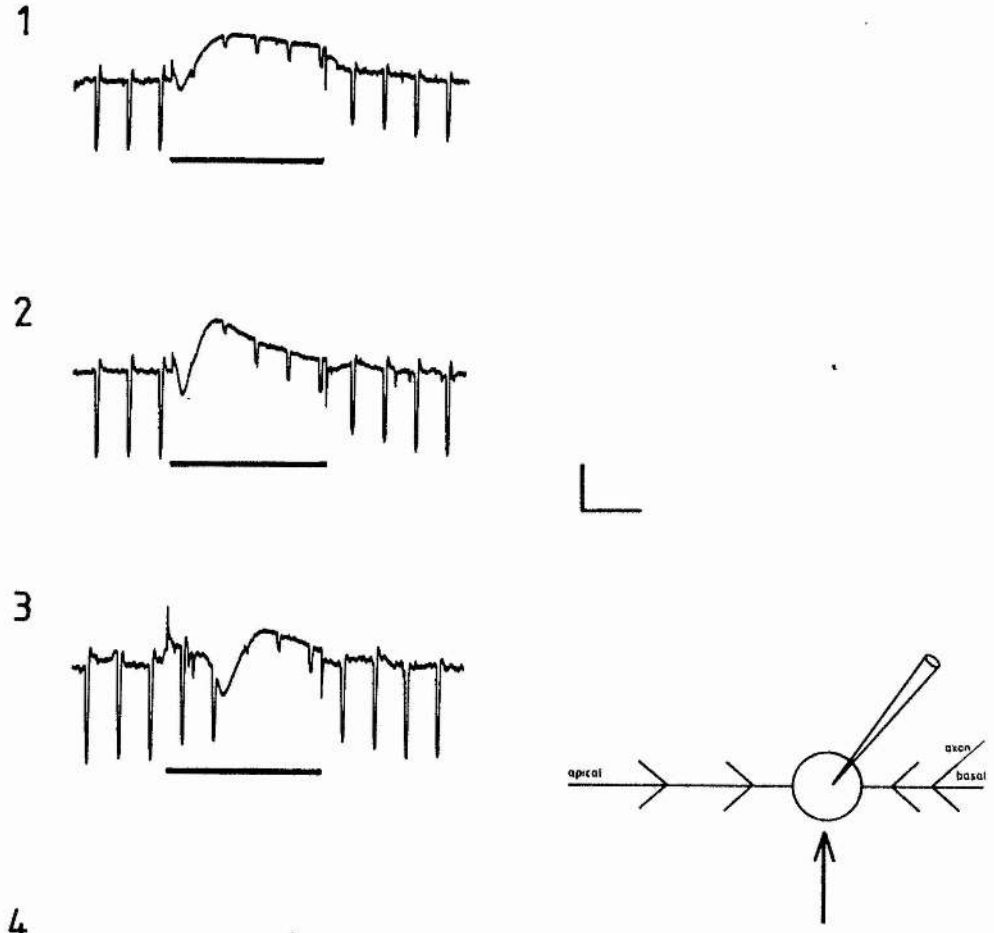
1. Control.
2. After 12 minutes in low $K^+$ ACSF.
3. After 16 minutes in low $K^+$ ACSF.
4. Wash.

$V_m$ -60mV.

Scale bars 5mV, 2s.

Note the increase in size of the hyperpolarizing response.

Diagram shows the position of iontophoresis.


1
2
3
4
apical
axon
basal

## 3.6. PHARMACOLOGY OF THE RESPONSES OF THE NEURONES TO GABA AND EDA.

### 3.6.1. PICROTOXIN.

#### 3.6.1.1. THE DEPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

Picrotoxin reduced the depolarizing response of the dendrites to GABA or EDA (both equally effectively) every time it was used (n=17). The threshold for this effect was 1μM. This value is higher than the $pA_2$ for picrotoxin in the rat cuneate nucleus (Simmonds, 1980). Picrotoxin did not always block the conductance change which accompanied the depolarizing response to the same extent that it reduced the voltage size of the depolarizing response. The effect of picrotoxin on the depolarizing response is shown in FIG 3.27. The size of the response was decreased to 33% of the size of the control response, the change in conductance being unaffected and remaining at 56% increase in conductance. Picrotoxin also reduced the depolarizing response of the dendrites to GABA (FIG 3.27.B). In this example the change in conductance accompanying the response was less in the blocked response (a 33% increase in conductance in the presence of picrotoxin compared to a 70% increase in conductance for the control response). Picrotoxin did not effect the reversal potential of the depolarizing response of the dendrites to GABA.

FIGURE 3.27.

Effect of picrotoxin (2μM) on responses of the dendrites to EDA and GABA.

A. EDA iontophoresis (40nA).

1. Control.

2. In the presence of picrotoxin. (16 min after switching the tap that controlled the solutions).

3. Wash.

$V_m$ -72mV.

Note the lack of effect of picrotoxin on the hyperpolarizing response.

B. GABA iontophoresis (60nA).

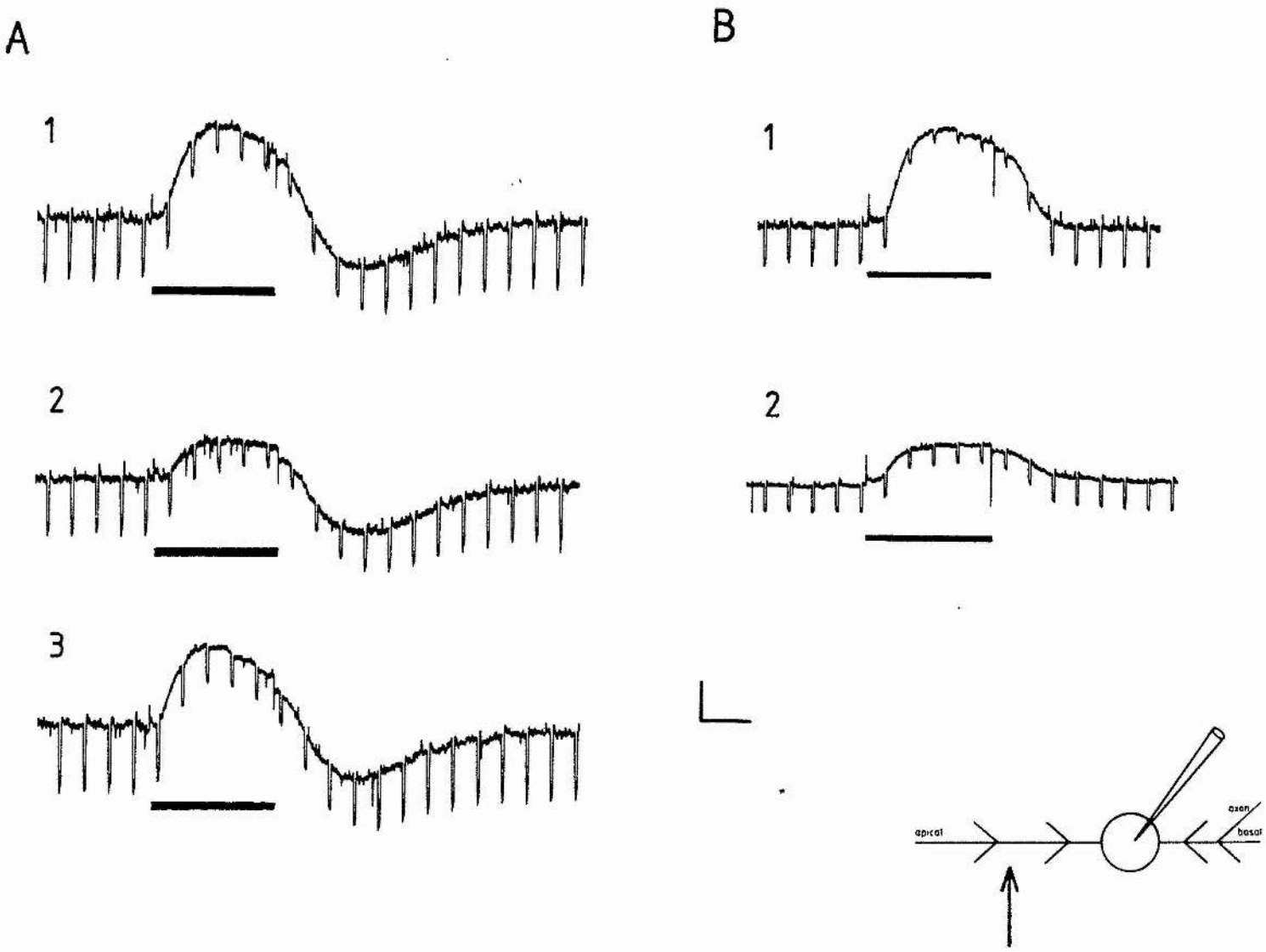
1. Control.

2. In the presence of picrotoxin. (12 min after switching the tap that controlled the solutions).

$V_m$ -60mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.


A
1
2
3
B
1
2
apical
axon
basal

The time course of block by picrotoxin (17μM) is shown in FIG 3.28. Typically, the size of the depolarizing response of the dendrites to EDA or GABA was reduced 10min after perfusion of picrotoxin had begun. Recovery of the depolarization to the size of the control responses took about 20min.

3.6.1.2. THE HYPERPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

The dendritic hyperpolarizing response to GABA (n=3) or EDA (n=6) was unaffected by picrotoxin, even when the concentration of picrotoxin was 20μM.

3.6.1.3. THE HYPERPOLARIZING RESPONSE OF THE CELL BODY TO GABA AND EDA.

Picrotoxin (2μM) partly blocked the hyperpolarizing response of the neurone body in one out of four neurones (FIG 3.29). In this single experiment, no recovery from the effect of picrotoxin was seen. However, the conductance change that accompanied the hyperpolarization of the cell body was reduced by the picrotoxin (from a 38% increase in conductance for the control response to a 27% increase in conductance in the presence of picrotoxin). Since the conductance changes of the hyperpolarizing response of the cell body were more reliable than the size of the response, and as the conductance change fully recovered (to a 40% increase in conductance) during the wash, the block seemed convincing. In the other three

FIGURE 3.28.

Time course of the block of the depolarizing response of the dendrites to GABA (40nA) by picrotoxin (17μM, applied between the arrows) and the recovery.

Note that the reduction in the size of the response may not have been maximal since the wash was started before the response size had levelled out.

Arrows show when the tap controlling the solutions was switched.

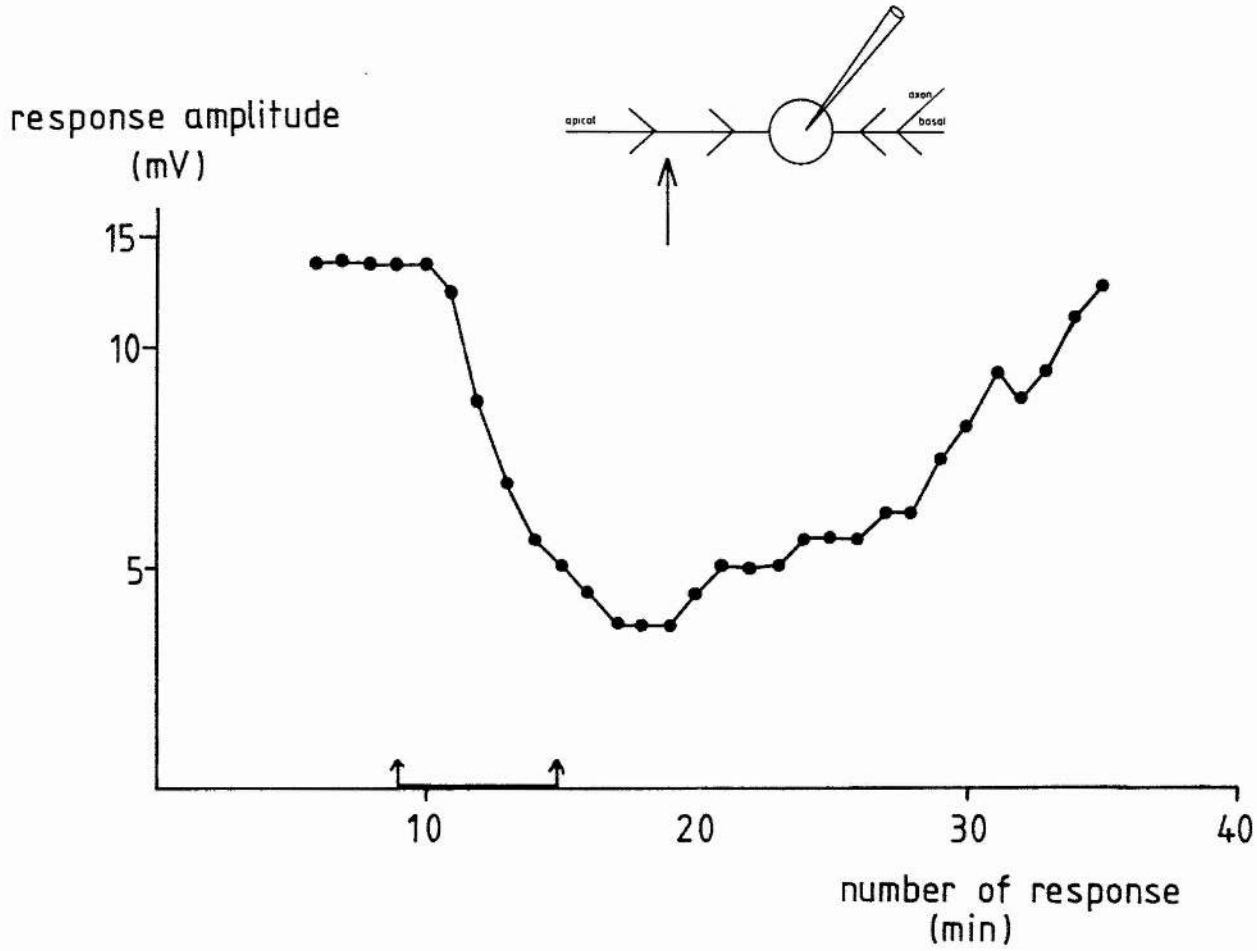

response amplitude
(mV)
15
10
5
apical
axon
basal
10
20
30
40
number of response
(min)

FIGURE 3.29.

Effect of picrotoxin (2μM) on the hyperpolarizing response of the cell body to GABA (30nA).

1. Control.
2. In the presence of picrotoxin, 16 min after switching the tap that controlled the solutions.
3. Partial wash, 36 min after switching the tap that controlled the solutions to normal ACSF.

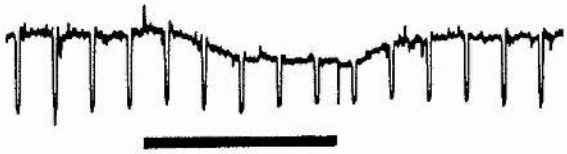
$V_m$ -55mV.

Scale bars 5mV, 2s.

Note the recovery of the conductance change after block by picrotoxin.

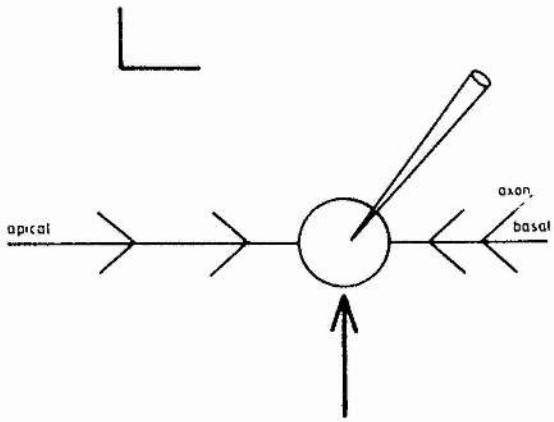
Diagram shows the position of iontophoresis.

1



2

3



experiments, the results were equally convincingly negative.

### 3.6.2. BICUCULLINE.

#### 3.6.2.1. THE DEPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

Two isomers of bicuculline were used - (+)bicuculline and (−)bicuculline methobromide. The latter was conveniently soluble in water. Both were equally effective at blocking the depolarizing response of the dendrites produced by GABA or EDA. It is unusual for both stereoisomers of an antagonist to be equally effective, although there are few reports of the selectivity of the isomers of bicuculline (see Introduction). The threshold concentration of bicuculline was 2μM. Three depolarizing responses of the dendrites to GABA are shown in FIG 3.30.A. One response was produced by an ejection current of 40nA, one response by 50nA and one response by 84nA, the last response being maximal. Depolarizing responses produced by these ejection currents after the neurone was exposed to (+)bicuculline (11μM) are shown below the control responses (FIG 3.30.B). The responses in bicuculline are reduced to about half of their control size. Since the depolarizing responses were paired (one control response and one response in the presence of bicuculline at a given iontophoretic current), the dose ratio can be estimated. In this example the dose ratio is about two. Similar data for depolarizing responses of the dendrites to EDA are shown graphically in FIG 3.31,

FIGURE 3.30.

Block of the depolarizing response of the dendrites to GABA by (+)bicuculline (11μM).

A. Control responses.

1. GABA 40nA.

2. GABA 50nA.

3. GABA 84nA.

B. In the presence of bicuculline, starting 14 min after switching the tap that controlled the solutions.

1. GABA 40nA.

2. GABA 50nA.

3. GABA 84nA.

$V_m$ -65mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.

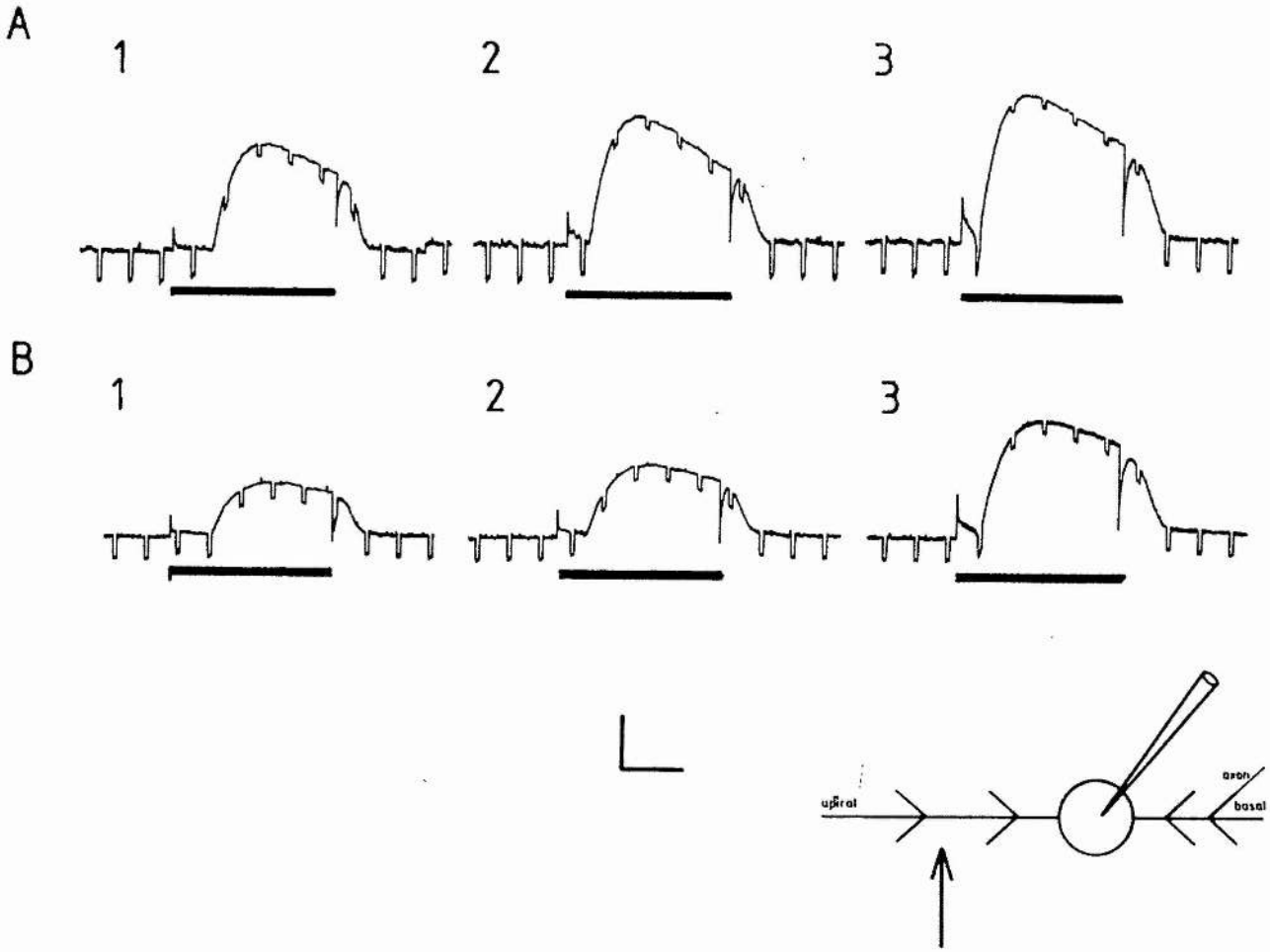

A
1
2
3
B
1
2
3
apical
axon
basal

FIGURE 3.31.

Dose response curve showing the effect of (-)bicuculline (11μM) on the depolarizing response of the dendrites to GABA.

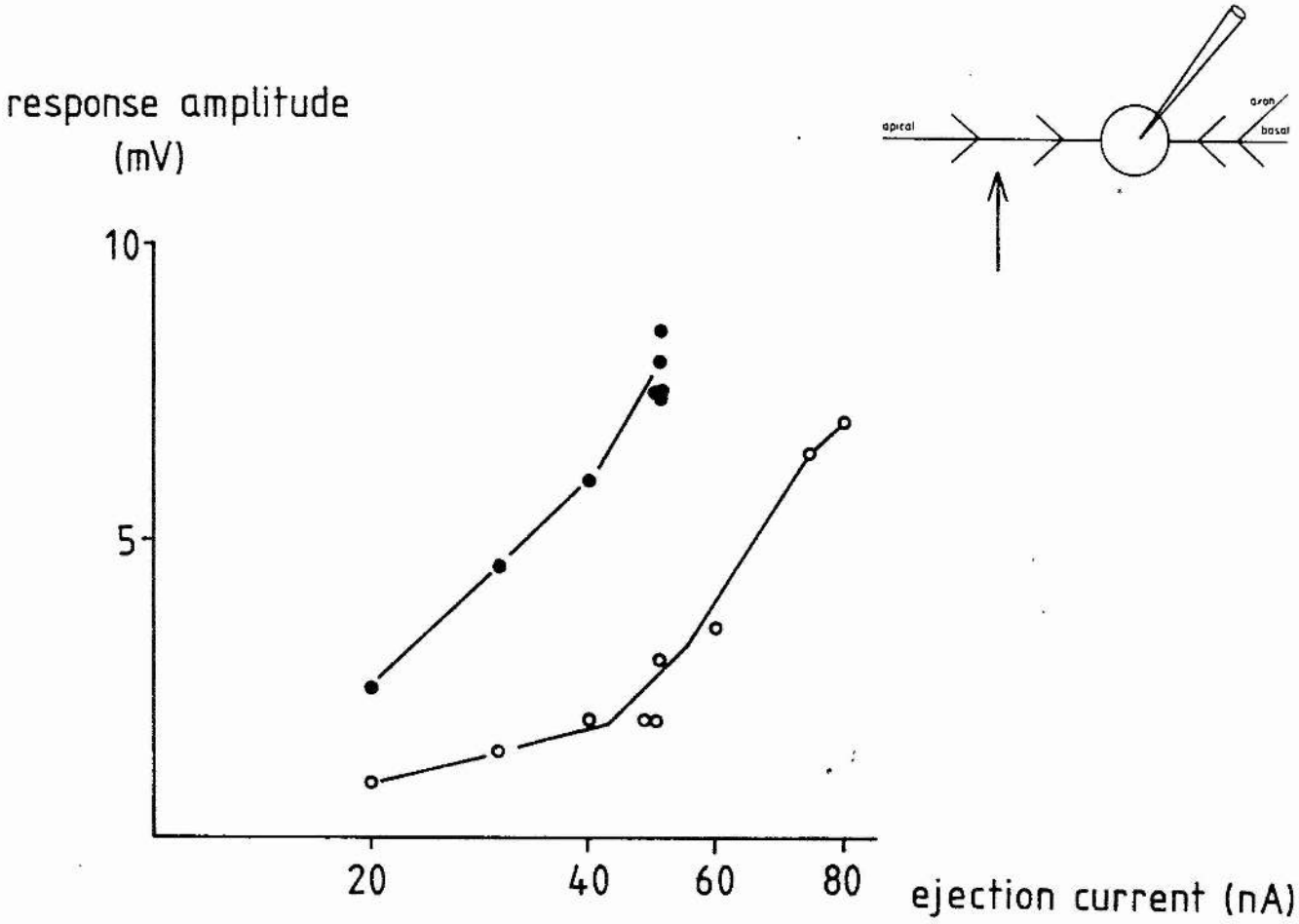
GABA iontophoresis.

Closed circles; control responses.

Open circles; responses in the presence of (-)bicuculline, starting 11 min after switching the tap that controlled the solutions.

Note that the curve is shifted to the right in a parallel fashion.

Diagram shows the position of iontophoresis.


response amplitude
(mV)
10
5
20
40
60
80
ejection current (nA)
apical
axon
basal

(+)bicuculline shifted the LDRC for EDA to the right. The dose ratio is about two. This concentration of bicuculline agrees with the $pA_2$ value for bicuculline in the rat cuneate nucleus (Simmonds, 1980).

3.6.2.2. THE HYPERPOLARIZING RESPONSE OF THE DENDRITES TO EDA.

Neither (+)bicuculline nor (−)bicuculline had any effect on the hyperpolarizing response of the dendrites to EDA. In FIG 2.32.A, the effect of three applications of EDA is shown. At the lowest iontophoretic current of 10nA, only a hyperpolarizing response of the dendrites was seen. This was accompanied by an increase in conductance of 22%. With an iontophoretic current of 30nA, a depolarizing response of the dendrites was seen superimposed on the hyperpolarizing response of the dendrites. The conductance increase accompanying these responses was 40%. With an iontophoretic current of 50nA, the depolarizing response of the dendrites was as large as the hyperpolarizing response of the dendrites. The conductance increase accompanying these responses was 50%. In the presence of bicuculline (11μM, FIG 3.32.B), the first response, a pure hyperpolarization, was unchanged. The conductance change remained a 22% increase. In the other two responses, however, the depolarizing phases of the response were not seen, and so the conductance changes associated with these depolarizations were also not seen. Thus the conductance increase for the second response (30nA) was reduced to 29%, and the conductance

FIGURE 3.32.

Block of the depolarizing response of the dendrites to EDA by (+)bicuculline (11μM) without an effect on the hyperpolarization.

A. Control responses.

1. EDA 10nA.

2. EDA 30nA.

3. EDA 50nA.

B. In the presence of (+)bicuculline, starting 12 min after switching the tap that controlled the solutions.

1. EDA 10nA.

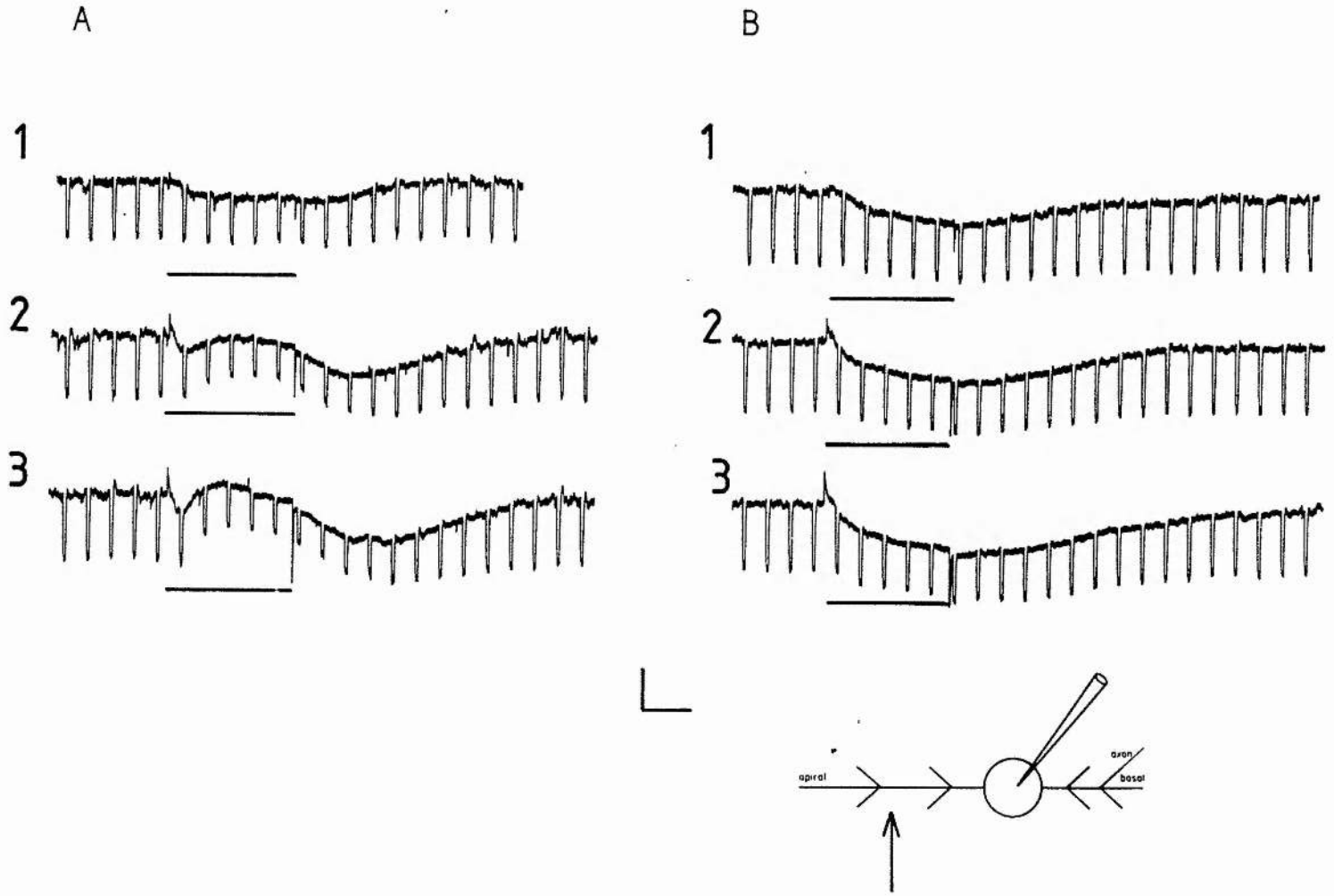
2. EDA 30nA

3. EDA 50nA.

$V_m$ -70mV.

Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.


A
B
1
2
3
1
2
3
apical
axon
basal

increase for the third response (50nA) was reduced to 41%. Since the conductance increases of the responses of the dendrites to EDA in bicuculline refer only to the hyperpolarization of the dendrites, the reversal potential of this response, uncontaminated by the depolarization of the dendrites, can be estimated by the method described by Assaf et al (1981). Taking data from FIG 3.32.B, the reversal potential was 16mV more negative than the membrane potential (ie -86mV in total).

3.6.2.3. THE HYPERPOLARIZING RESPONSE OF THE CELL BODY TO GABA.

The effect of bicuculline on the cell body hyperpolarization was only tested in 2 neurones. On both occasions (-)bicuculline (2μM) partly blocked the response. No higher concentrations were used.

3.6.3. THE BENZODIAZEPINES.

Five drugs in this class were used: diazepam, flurazepam, clonazepam, lorazepam and nitrazepam. The first two were used most frequently.

3.6.3.1. THE DEPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

Clonazepam (20μM, n=2), nitrazepam (27μM, n=4) and lorazepam (25μM, n=1) had no effect on the depolarizing

response of the dendrites produced by GABA. The solubility of these compounds in water is very poor (Merck Index, edition 9, 1976. pp304,726,853). To test the solubility of clonazepam in saline solution, a double beam spectrophotometer was used to compare solutions in ethanol and in saline. A solution of clonazepam, theoretically at a concentration of 3.8mg/500ml appeared to have a concentration of 2.6mg/500ml. However, the soluble concentration in the experiments would still have been reasonably high.

Both diazepam (2μM-14μM) and flurazepam (2μM-30μM) potentiated the depolarizing response of the dendrites to GABA and EDA, whether or not the response was desensitized. This potentiation occurred in nine neurones out of 15 (diazepam) and nine neurones out of 20 (flurazepam). Of the remainder, diazepam reduced the response in two neurones and had no effect in four neurones. Flurazepam reduced the response in two neurones and had no effect in nine neurones. Diazepam and flurazepam had opposite actions in 2 neurones tested with both compounds. Here, flurazepam (10μM) reduced the response and diazepam (10μM) potentiated the response (FIG 3.8). The effect of flurazepam (10 and 30μM) is shown in FIG 3.33. Flurazepam (10uM) potentiated the depolarizing response of the dendrites to GABA by 77% with an increase in the conductance change from 25% to 50%. Flurazepam (30μM) potentiated the depolarizing response of the dendrites to GABA by 75%. There was, however, no clear dose dependency of the effects of the benzodiazepines.

FIGURE 3.33.

Potentiation of depolarizing responses of the dendrites to GABA by flurazepam.

A. GABA (30nA), flurazepam 30μM.

1. Control responses.

2. Responses in the presence of flurazepam, starting 16 min after switching the tap that controlled the solutions.

3. Wash.

$V_m$ -70mV.

B. GABA (50nA), flurazepam 10μM.

1. Control.

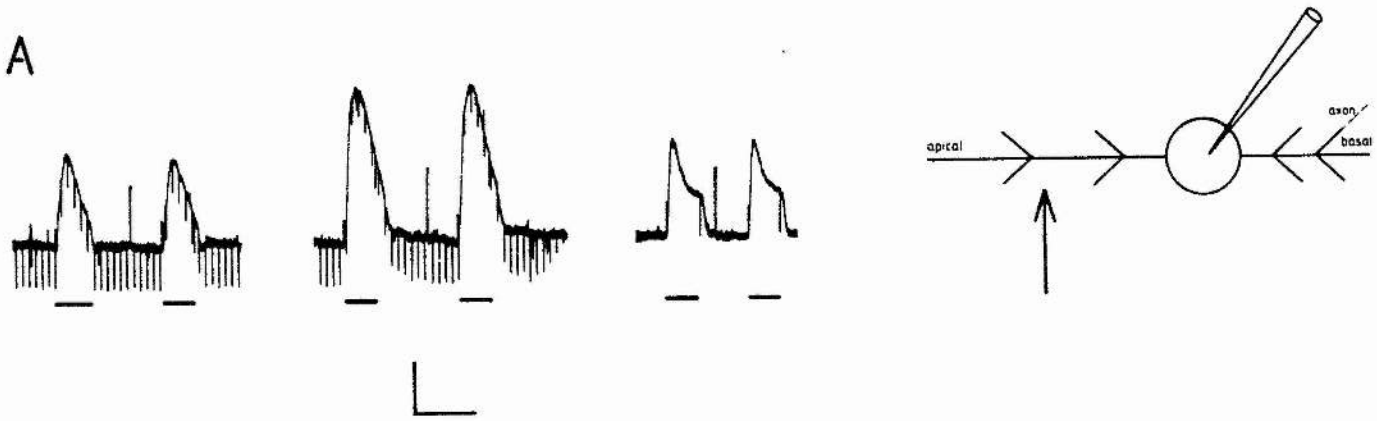
2. In the presence of flurazepam, starting 22 min after switching the tap that controlled the solution.

3. Wash.

$V_m$ -70mV.

Scale bars 5mV. 10s.

Diagram shows the position of iontphoresis.


A
apical
axon
basal
B

EDA was only used twice with diazepam (10μM); the depolarizing response of the dendrites was potentiated. The data are shown graphically in FIG 3.34. The LDRC for EDA was shifted to the left in the presence of diazepam.

3.6.3.3. THE HYPERPOLARIZING RESPONSE OF THE DENDRITES TO EDA.

None of the benzodiazepines had any effect on the hyperpolarization, even when the depolarizing response of the dendrites was potentiated.

3.6.3.4. THE HYPERPOLARIZING RESPONSE OF THE CELL BODY TO GABA AND EDA.

The hyperpolarization of the cell body produced by GABA was potentiated 100% by flurazepam (30μM, n=1) (FIG 3.35) but was unaffected by diazepam (10μM, n=2). The data from both the hyperpolarizing response of the dendrites to EDA and the hyperpolarizing response of the cell body to GABA is sparse.

3.6.4. IS THERE A CHLORIDE PUMP IN THE DENDRITES?

Ammonium ions are reported to block hyperpolarizing inhibition in the spinal cord (Lux, 1971). Ammonium chloride (2mM) had no effect on the depolarizing response of the dendrites to GABA, other than that expected from the irreversible depolarization of the neurone that ammonium chloride produced.

FIGURE 3.34.

Potentiation of the depolarizing response of the dendrites to EDA by diazepam (10μM).

Closed circles; control responses.

Open circles; responses in the presence of diazepam, starting 26 min after switching the tap that controlled the solutions.

Crosses; wash.

$V_m$ -70mV.

Diagram shows the position of iontophoresis.

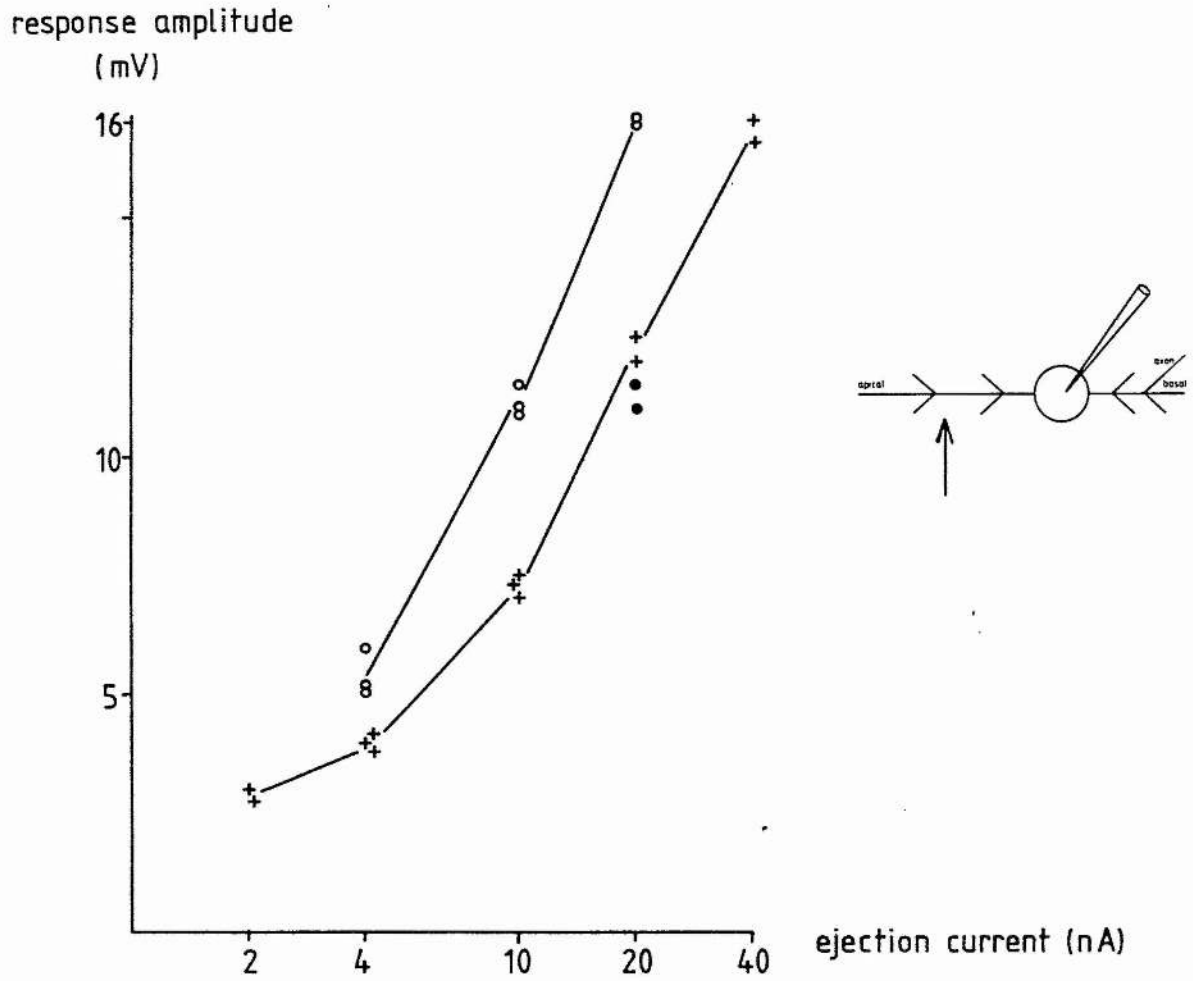

response amplitude
(mV)
16
10
5
2
4
10
20
40
ejection current (nA)
apical
basal

FIGURE 3.35.

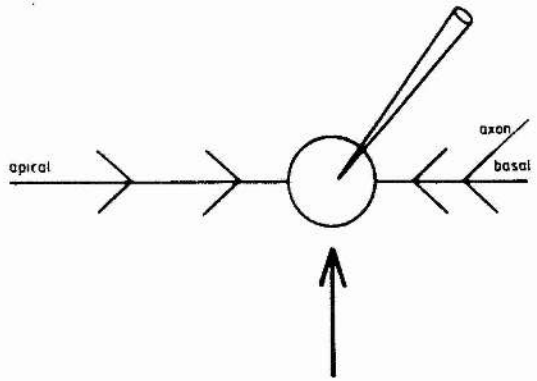
Potentiation of the hyperpolarizing response of the cell body to GABA (20nA) by flurazepam (30μM).

1. Control.
2. Response in the presence of flurazepam, 18 min after switching the tap that controlled the solutions.

$V_m$ -60mV.

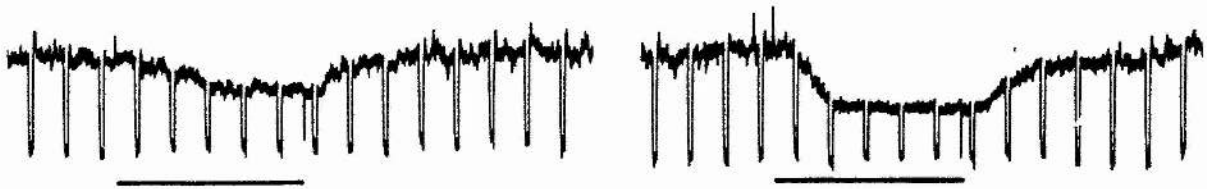
Scale bars 5mV, 2s.

Diagram shows the position of iontophoresis.




1

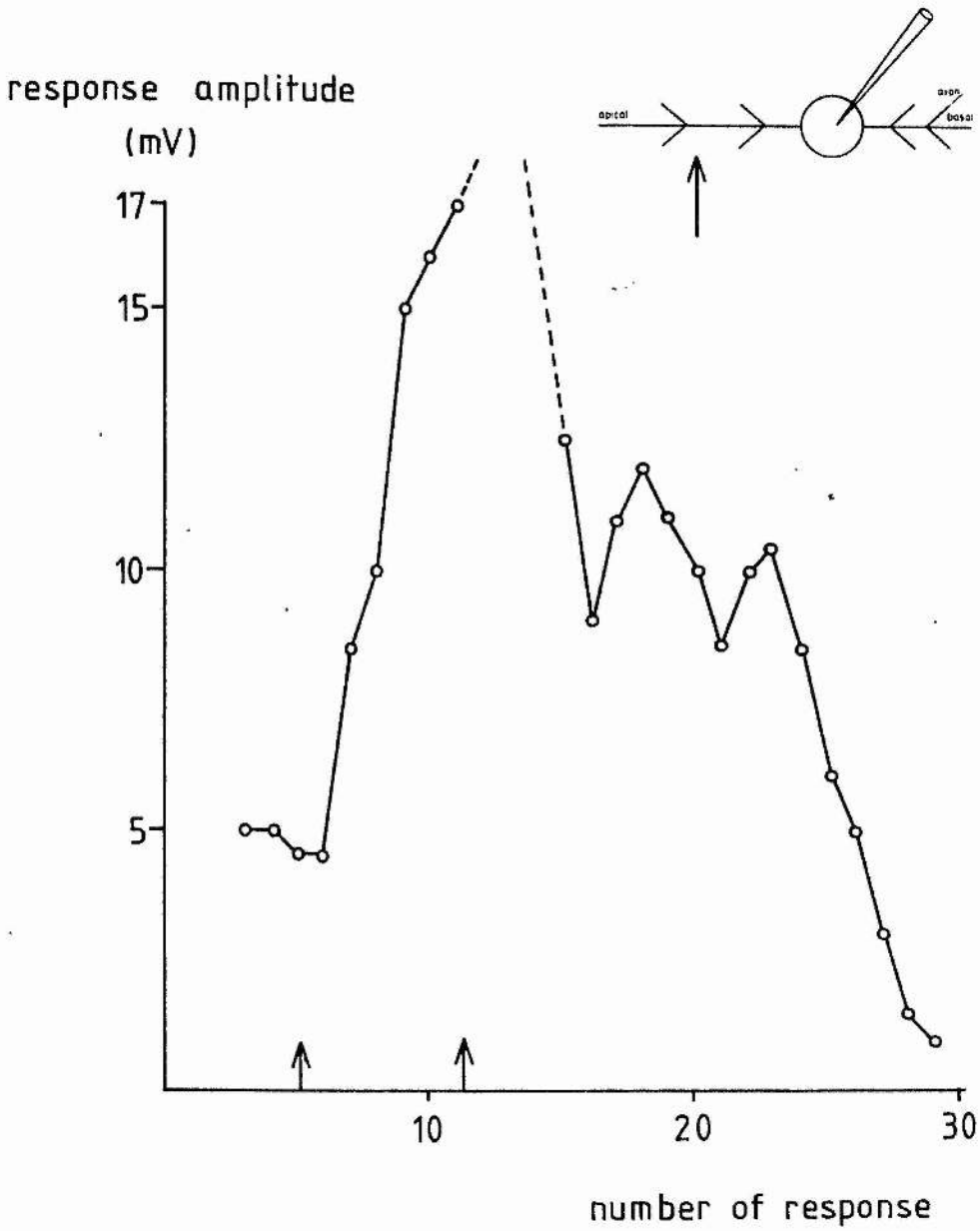
2



Two drugs were used to test for the presence of a $Cl^-$ transport system in the dendrites that might support the depolarizing response. The effect of frusemide and SITS was tested on the depolarizing response of the dendrites to GABA and on the reversal potential of this response. Frusemide (100μM) made the reversal potential of the response less negative in one experiment (n=8). SITS (11-38μM) reduced the depolarization at the highest concentration but had no effect at lower concentrations apart from a small increase in membrane potential. There was no effect of SITS on the hyperpolarizing response of the cell body produced by GABA. In one experiment a more complex design was used. After reproducible depolarizing responses of the dendrites to GABA were established, the slice was perfused in low $Cl^-$ ACSF containing SITS (20μM) in order to deplete the dendrites of $Cl^-$. SITS was also present in the ACSF (normal $[Cl^-]$) to prevent $Cl^-$ reentering the dendrites during washout. As FIG 3.36 shows, this was what appeared to happen. The responses in low $Cl^-$ ACSF, initially larger than the controls, as expected, fell to just above control levels. During the wash, the responses became even smaller. This is consistent with a depletion of $Cl^-$ from the dendrites.

FIGURE 3.36.

Effect of chloride depletion from the dendrites on the depolarizing response of the dendrites to GABA. Low $Cl^-$ ACSF (88mM $Cl^-$) and SITS (10μM) were perfused between the arrows. The subsequent wash also contained SITS (10μM).
Arrows show when the tap that controlled the solutions was switched.


response amplitude
(mV)
17
15
10
5
10
20
30
number of response
apical
basal

# CHAPTER FOUR

## DISCUSSION OF THE RESULTS.

## 4.1. THE HIPPOCAMPAL SLICE.

The hippocampal slice was a useful preparation in that recordings of several hours duration could be made from pyramidal neurones and that experiments were successfully done on these neurones.

Although it was possible to cut slices of 50μm thickness on the Vibratome, 300μm slices were viable for longer because the neurones in the middle of the slice were undamaged and because the slice was thick enough to support itself. Thin slices were damaged by the net which supported them. The protocol of cutting slices was arranged round the need to avoid thermal shocks to the tissue. Although some workers cut slices in ice-cold ACSF and store the slices at various temperatures, cold-cut or cold-stored slices in this study were not as viable as slices cut from tissue that had been carefully cooled to 33°C from body temperature.

It is necessary to justify the making of intracellular recordings at 33°C. Although receptor characteristics have been observed to change with temperature, usually cooling to less than 30°C is required. It was hoped that the GABA and benzodiazepine receptors (both of which are studied in binding assays at between 4°C and 37°C) were unaffected at the temperature used here. The change in temperature from 37° to 33° changes $E_K$ by about +3mV.

Apart from these esoteric considerations, slices are maintained at 30°C to 35°C by other workers, and so a comparison between my results and their results is valid on this count at least. In addition, at 37°C, the slices became useless within 2h.

Pyramidal neurones *in vivo* at 37°C (Kandel et al, 1961) have much the same properties as those in this study (FIG 3.1) and those reported for hippocampal slices at 37°C (Schwatzkroin, 1975). One difference between the observations *in vivo* and *in vitro* is that, in intact animals, pyramidal neurones in the hippocampus fire action potentials at 2-8Hz, while active neurones are rare in slices and most neurones did not fire at all in the present study. It is possible that impaled neurones were hyperpolarized away from threshold by leakage of $K^+$ from the electrode. Since few synaptic potentials were seen, it is likely that excitatory inputs to the neurones were not active. In some cases, of course, the inputs to the hippocampus had been severed during slicing but the excitatory pathway from CA3 neurones to CA1 neurones (the Schaffer collateral pathway) was intact, as activation of this pathway by stimulation produced EPSPs and spikes in the pyramidal neurones (FIG 3.2.1). Apart from excitatory inputs, inhibitory potentials were occasionally recorded spontaneously (FIG 3.2.3). These IPSPs probably arose from basket neurone activity. Basket neurones are activated by collaterals from CA1 neurones and although the impaled neurone was not active,

other neurones may have been spiking and exciting the basket neurones, thus producing IPSPs in the neurone from which the recording was made. Basket neurones synapse onto the dendrites and cell body of CA1 pyramidal neurones and probably release GABA, which acts on the CA1 neuronal membrane to produce hyperpolarizing IPSPs. This recurrent inhibition includes neurones other than the one which is active, since the basket neurone projections reach several hundred micrometres both within the lammellae and laterally. Further evidence for an intact neuronal circuit in the slices was provided by the results of intracellular stimulation.

Making the neurone fire by injecting depolarizing current intracellularly activated the recurrent inhibition via the axon collateral and basket neurones, and the conductance of the pyramidal neurone membrane decreased and the neurone hyperpolarized. This is a similar effect to the IPSP, though the inhibition was much more prolonged than a single IPSP. There was unfortunately no time to examine the pharmacology of the recurrent inhibition or the ionic mechanism of the response of the pyramidal neurone. The hyperpolarization and conductance change may have been due to a $Ca^{++}$-dependent $K^{+}$ conductance (Haas and Konnerth, 1983). Recurrent inhibition and this conductance might be distinguished pharmacologically. The increase in conductance and the hyperpolarization were only seen in neurones in slices from the lateral parts of the hippocampus. These slices were cut at an angle oblique to the

axis of the hippocampus (see Methods). The recurrent inhibition is known to be arranged in a similar angle to the axis of the hippocampus. The recurrent inhibitory pathway may therefore have been intact in the more lateral slices, which would explain why the effects of intracellular stimulation were only seen in the lateral slices. These observations provide circumstantial evidence that the hyperpolarization and conductance increase seen after intracellular stimulation were due to recurrent inhibition.

In addition to synaptic activity, small amplitude spikes were sometimes seen, usually just after an application of GABA to the dendrites. These spikes were of much shorter duration than EPSPs. It is possible that they were action potentials in the dendrites which were decrementally conducted to the cell body. If this is the case, then the dendrites appear not to have regenerative action potential processes, since the small amplitude (2mV) of the spikes suggests that the dendrites attenuate signals passing along them. This assumes that the spikes are larger in the dendrites than they were seen to be in the cell body (see Introduction).

The membrane potential of the pyramidal neurone could be changed by perfusing the slice with low $K^+$ ACSF. The concentration of $K^+$ used here, 0.63mM compared to 3.75mM in normal ACSF, was calculated to produce a change in the equilibrium potential for $K^+$ of over 50mV more negative than

$E_K$ in normal ACSF. If the neuronal membrane was affected fully by such a change, then a hyperpolarization of 50mV would have resulted. As expected, however, the membrane did not behave in this way, since it was not a perfect potassium electrode. The behaviour of the neuronal membrane was useful in the present study. When the slice was perfused with the low $K^+$ ACSF, the neurone hyperpolarized by about 5mV. Any response that was $K^+$ dependent was greatly increased in size because the ionic gradient for the response was larger. The effect of low $K^+$ ACSF on the responses of the pyramidal neurone to GABA or EDA is discussed below.

The current/voltage relationship of the neurone was not linear. At hyperpolarized levels, the slope resistance was decreased. Since this reduction in membrane resistance might have reduced the size of constant current hyperpolarizing pulses, the pulses were no larger than was necessary in order to measure them accurately. The size of the constant current hyperpolarizing pulses changed during a response. When GABA or EDA was applied, the pulses became smaller. This indicates that the resistance of the neuronal membrane had fallen. The shape of the bottom of the hyperpolarizing pulses (the envelope) show the shape of the response at the hyperpolarized level which the pulses reach, and this envelope contributes to the decrease in resistance that the pulse size also shows. The change in resistance measured was the change in input resistance of the neurone, not the change in membrane

resistance. The two are related, but the relationship is complicated by the geometry of the neurone.

The membrane potential was measured when the electrode was withdrawn from the neurone. Althought the concentration of ions in the electrode was constant, intracellular and extracellular fluids have widely different compositions. The junction potential between the electrode when inside the neurone and the junction potential when the electrode is outside the neurone was therefore different. No compensation for the change in junction potential has been made in the measurement of membrane potential. All changes in membrane potential during an experiment were related to the final value when the electrode was withdrawn.

## 4.2. RESPONSES OF THE NEURONES TO GABA AND EDA.

### 4.2.1. PROPERTIES AND PHYSIOLOGICAL ROLE OF RESPONSES OF THE DENDRITES TO GABA AND EDA.

EDA has been reported to release GABA from brain slices (Forster et al, 1981). It is therefore possible that responses to EDA are mediated by GABA. If, as Alger and Nicoll (1982) suggest, the hyperpolarizing response of the dendrites to GABA and THIP is the synaptic response, then EDA might be expected to produce this response if it releases GABA from nerve terminals; this released GABA could then act on postsynaptic receptors to produce the hyperpolarization of the dendrites of the pyramidal neurone. If this is the case, why then was GABA ineffective in producing the hyperpolarizing response of the dendrites? It may be that the GABA-ergic synapses are not usually open to exogenous GABA, but EDA can nevertheless cause the release of GABA at these synapses. It is likely that the hyperpolarizing response of the dendrites to EDA was an indirect effect of EDA on GABA receptors. The depolarizing response of the dendrites to GABA and EDA was reliably found and therefore easy to work with. The existence in the dendrites of this depolarizing response suggests several questions. For example, if the hyperpolarizing response of the dendrites is the synaptic response, what then is the function of the depolarizing response? Alger and Nicoll (1982) suggest that receptors which mediate the depolarizing response to GABA are extrasynaptic and are activated by overspill from

GABA-ergic synapses. This might explain the change in polarity of the IPSP which occurs when hippocampal neurones are strongly stimulated, or are affected by drugs such as the barbiturates. Whether such a situation has any physiological significance is a matter for speculation, but several observations can be made. In this study no inhibitory, depolarizing potentials were recorded when the neurone was stimulated antidromically or orthodromically. However, the depolarizing response of the dendrites to iontophoresed GABA sometimes caused the neurone to fire action potentials. Since the conductance change occurs at the site of the response only, one can guess that the conductance change does not inhibit distant dendrites. The depolarization of the dendrites would have been more widespread than the conductance increase and may have activated the neurone some distance from the site of the response. This effect would have been limited by the electrotonic parameters of the neurone (see Introduction). GABA is reported to have an excitatory action in vivo (Obata, 1976). Clearly GABA could excite neurones in this study, but only in special circumstances. These circumstances are unlikely to be met in vivo.

Perhaps the depolarizing response and the hyperpolarizing response of the dendrites interact in a more subtle way than inhibition vs. excitation. Small depolarizing responses of the dendrites to GABA were less effective at reducing the firing rate of neurones than small hyperpolarizing responses of

the dendrites to GABA (FIG 3.11). Thus the effect of great activity across the GABA synapses in the dendrites, producing great inhibition, would be modified by a depolarization resulting from the overspill of GABA. This would be difficult to test directly, but the effect of GABA uptake inhibitors on IPSPs produced by antidromic stimulation of the pyramidal neurones might show the shift in polarity of the hyperpolarizing IPSP to the depolarizing IPSP at a lower frequency of stimulation than in the absence of the drug. The excitatory effect of stimulation of the Schaeffer collaterals could be tested during both depolarizing and hyperpolarizing inhibition.

It is perhaps too easy to dismiss the presence of a depolarization in the dendrites merely as imperfect or incomplete evolution.

### 4.2.2. THE PROPERTIES AND PHYSIOLOGICAL ROLE OF THE RESPONSES OF THE CELL BODY TO GABA AND EDA.

Most of the synapses of the basket neurones are found on the cell body of pyramidal neurones. It is likely that the response of the cell body to GABA is the response to the transmitter at these synapses. Both the response to GABA and the IPSP are hyperpolarizing. Alger and Nicoll (1982) report that THIP hyperpolarizes the cell body. The hyperpolarizing response of the cell body to GABA was often small, and sometimes invisible except for the increase in conductance that

accompanied the response. The time course of the hyperpolarizing response of the cell body to GABA was different to the response produced by EDA. The shape of the response to GABA was almost square, with rapid onset and recovery of the potential change and conductance increase. The hyperpolarizing response of the cell body produced by EDA was invariably slower in onset and recovery. This is consistent with the hypothesis that EDA releases GABA; in this case, presumably release from nerve terminals on the cell body of the pyramidal neurone. The hyperpolarizing response of the cell body to both GABA and EDA was clearly inhibitory; both the polarity of the responses and the increase in conductance which accompanied them would keep the cell body away from the threshhold for firing. The reversal potential of the hyperpolarizing response of the cell body to GABA or EDA was between 0mV and 10mV more negative than the membrane potential, while the reversal potential of the hyperpolarizing response of the dendrites to GABA or EDA was 16mV more negative than the membrane potential of the cell body. This difference says more about the inaccuracy of obtaining the reversal potential of a dendritic response than it does about the difference between the hyperpolarizing responses.

### 4.2.3. THE EFFECT OF LOW $Na^+$ ACSF ON THE RESPONSES OF THE DENDRITES TO GABA AND EDA.

Another question raised by the presence of two responses to GABA in the dendrites concerns the ionic mechanism

underlying the responses. Clearly, both the depolarizing response of the dendrites and the hyperpolarizing response of the dendrites could not have the same ionic mechanism except under unusual circumstances, although they could share a common conductance if more than one ion were involved in both responses.

The effect of $Na^+$ substitution on the depolarizing response of the dendrites to GABA was the opposite of the effect expected if the response was $Na^+$ dependent. The depolarization increased in size in low $Na^+$ ACSF. It is known that GABA uptake can limit the size of responses to GABA even when GABA is applied iontophoretically. Alger and Nicoll (1982) show this in their FIG 11, where $Na^+$ substitution to 15mM $Na^+$ produces an increase in the size and duration of the depolarizing response of the dendrites to GABA. In fact, this figure looks like FIG 3.20B and C in this study. The effect of low $Na^+$ ACSF, although suprising, is fairly easy to explain as a block of GABA uptake. If, however, the depolarizing response depends on a $Na^+$ conductance, then the reduction in $[Na^+]_o$ would decrease the size of the response.

The normal $[Na^+]_o$ was 150mM. On reduction to 26mM, the $E_{Na}$ becomes less positive by about 40mV, but still remains positive. This change in $E_{Na}$ is relatively small compared to the potential difference between the membrane potential and $E_{Na}$, and so even this large a change in the

$[Na^+]_o$ would have had relatively small effects on a $Na^+$-dependent depolarizing response; perhaps producing a reduction of one third for a small response. Even this change is more than expected for the depolarizing response of the dendrites to GABA since this response was also partly $Cl^-$ dependent. Furthermore, low $Na^+$ ACSF increased the input resistance of the neurone by about 10%. This would increase the size of the depolarizing response to GABA. For these reasons, it is not surprising that $Na^+$ substitution did not clarify the involvement of $Na^+$ in the depolarizing response of the dendrites to GABA.

Reductions of $[Na^+]_o$ below 26mM were not possible without radical changes in the buffering system of the ACSF. Since $Cl^-$ gradients may be linked to buffering by $HCO_3^-$ (Aickin and Brading, 1982), and EDA depends on $HCO_3^-$ for its mimicry of GABA (Hill, 1983) and GABA receptor binding is different in different buffering systems (Bowery, Hill, Hudson, Perkins and Stone, 1983), it was decided not to add yet another variable to the experiments by changing the buffer.

Although the depolarizing response of the dendrites to GABA was increased in low $Na^+$ ACSF, the depolarizing response of the dendrites to EDA was decreased. This is consistent with involvement of $Na^+$ in the response. According to the argument put forward above, however, the depolarizing response of the dendrites to EDA should have been little changed by the

level of $Na^+$ substitution. The effect of low $Na^+$ ACSF can also be explained in another way. If the depolarizing responses to EDA are indirect (ie EDA releases GABA which then acts on the dendrites) in the same way as the hyperpolarizing response of the dendrites to EDA seems to be, then a reduction in $[Na^+]_o$ would tend to reduce this release since the mechanism by which EDA releases GABA is $Na^+$ dependent (Forster et al, 1981). Since EDA blocks GABA uptake also by a $Na^+$ dependent mechanism, low $Na^+$ ACSF would also reduce the uptake of released GABA. This would explain the change in shape of the depolarizing response of the dendrites in low $Na^+$ ACSF since the response did not fade during the application of EDA as did the control response (FIG 3.20.A.2). The responses in FIG 3.20.A also show that a small hyperpolarizing response was present in the experiment. In low $Na^+$ ACSF, the response was smaller but more prolonged. This is consistent with the hyperpolarizing response of the dendrites to EDA being mediated by a release of GABA by a $Na^+$ mechanism.

For the hypothesis that EDA releases GABA which then acts on the dendrites to be valid, there must be enough GABA present in the slice to be released every minute for several hours, as reproducible responses to EDA could be obtained over this period. There is a considerable amount of GABA in neurones and also in glial cells (which also have a $Na^+$ dependent uptake mechanism for GABA) since the synthetic pathway for GABA (and

onwards) represents about 10% of glucose oxidation. It is possible that EDA releases GABA from nerve terminals (to produce the hyperpolarizing response of the dendrites) and from non-neuronal cells (to produce the extrasynaptic depolarizing response of the dendrites). There is, however, no way of ruling out a direct mimetic effect of EDA on GABA receptors except pharmacologically.

In favour of the release hypothesis is the observation in this study that GABA and EDA are equipotent in producing the depolarizing response of the dendrites, while in the rat cervical ganglion, where EDA apparently acts directly, EDA was 20x less potent than GABA (Perkins et al , 1981). As expected from this low potency, the depolarization of the ganglion that EDA produced was blocked more effectively by bicuculline than was the depolarizing response of the ganglion to GABA. In the present experiments, (+) or (-) bicuculline reduced the depolarizing response to EDA and to GABA equally effectively , which suggests that bicuculline blocked a response to GABA even when EDA was iontophoresed, since it is very unlikely that different agonists have the same affinity for a single receptor. Against the release hypothesis is the very similar time course of the depolarizing responses of the dendrites to GABA and EDA. It is conceivable that GABA could be released by EDA and reach the receptors which mediate the depolarizing response in the same time and at the same rate that iontophoresis of GABA supplies GABA to the receptors, but this

seems unlikely. If EDA released GABA and blocked the re-uptake of the GABA, it is unlikely that the depolarizing response of the dendrites to EDA would fade in the same way as the response to GABA faded; in practice, the depolarizing responses produced by GABA and EDA were often indistinguishable.

The release hypothesis could be tested by applying GABA and EDA to the dendrites in the presence of an uptake inhibitor such as nipecotic acid. GABA responses would then not be limited in size by uptake and the direct effects of EDA and its potency relative to GABA might be clearer. Another test would be to examine the effect of EDA on neurones without synaptic inputs (such as dorsal root ganglion neurones, which are depolarized by GABA) and without a glial or other non-neuronal supply of GABA. Neuronal cultures or autonomic ganglia might also be suitable, especially if GAD, the enzyme that synthesises GABA, was blocked by a drug such as amino oxyacetic acid. Spinal cord neurones in culture do not respond to EDA, even when a response to GABA is present (P.W. McCarthy, personal communication). A further experiment would be to load slices with labelled GABA and measure any increase in GABA efflux produced by the iontophoresis of EDA, although the assay might not be sensitive enough to detect the small quantities involved.

Unfortunately, the effect of low $Na^+$ ACSF on hyperpolarizing responses of the cell body to EDA or GABA was

not examined. Since the hyperpolarization would not have been mediated by $Na^+$, such an experiment might have elucidated the role of uptake and release mechanisms involved in the response to EDA.

4.2.4. EFFECT OF IONS OTHER THAN SODIUM ON THE DEPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

The depolarizing response of the dendrites to GABA and EDA was clearly chloride dependent, because the size of the responses increased when the slice was perfused with low $Cl^-$ ACSF. As this observation alone could not distinguish all the conductances in the response, and as the effects of low $Na^+$ ACSF were so complex, a quantitative experiment was attempted. The method (an extension of that described by Assaf et al , 1981) is described in the Methods chapter. The method depends on an accurate estimate of reversal potential. The reversal potential measured in normal ACSF with the method of Assaf et al (1981) was similar to the reversal potential measured by displacement of the cell body membrane potential (compare FIG 3.21 with Table 3.). This latter measurement was not often possible because the depolarizing response of the dendrites to GABA and EDA was not usually susceptible to current injection at the cell body. The method of Assaf et al (1981) produced results but the many limitations of the experiments precluded any worthwhile analysis.

The limitations are the result of the electrotonic

properties of the neurones. These properties for CA1 pyramidal neurones have been examined by Turner and Schwartzkroin (1980), Traub and Llinas (1979) and Brown, Fricke and Perkel, (1981). The conclusions are much the same, but as the analysis of Turner and Schwartzkroin (1980) includes a useful discussion of the present problem, this paper is refered to below.

Using the equations of Rall (1959), Turner and Schwartzkroin (1980) report that the length constant of the dendrites is about 1.5 space constants, which results in only 1% of voltage changes in the distal dendrites being transferred to the cell body. Current transfer from the distal dendrites is about 20% and from the stratum radiatum dendrites is about 50%. These results suggest that responses to GABA obtained in the stratum radiatum dendrites and measured in the cell body in the present study were attenuated, and that the voltage deflection produced by current injected into the cell body in the form of hyperpolarizing pulses or DC current would only be slightly affected by conductance changes in the dendrites. These theoretical considerations would produce the following limitations in the method of Assaf et al:

1. The conductance ratio measured at the cell body would be larger than if it were measured at the site of the response. This leads to the reversal potential of the response being measured as less negative than the actual reversal potential.

2. The voltage change of the response would be decrementally conducted to the cell body, leading to the

measurement of the reversal potential of the response as being more negative than the actual response. A further critisism of the method is the use of concentrations in the Nernst equation instead of activities. The activity of $Cl^-$ may be different outside and inside the neurone (Motokizawa, Reuben and Grundfest, 1969). It has been assumed that the reduction in $[Cl^-]$ in the ACSF reduced the $[Cl^-]$ by the same amount at the outer surface of the neuronal membrane.

That the elecrotonic parameters of the neurone may limit the the depolarizing response of the dendrites is suggested by the experiment shown in FIG 3.5. It has been assumed that changes in the external concentrations of ions do not affect intracellular concentrations.

The changes in reversal potential of the depolarizing response of the dendrites to GABA produced by low $Cl^-$ ACSF were between 5mV and 11mV. It is not possible to state convincingly whether these values are overestimates or underestimates.

In FIG 3.5.B. three responses of the same size are shown. They are all depolarizing responses of the dendrites to GABA, but come from three different sites. The iontophoresis current was carefully adjusted so that the responses were the same size, and the position of iontophoresis carefully selected. As can be seen from the figure, the conductance changes associated

with the responses are not the same. The response most distant from the cell body shows the least change in conductance, and the response nearest the cell body shows the largest increase in conductance. From this experiment, the conductance ratio of distant responses was underestimated. This leads to the conclusion that the changes in reversal potential measured by the method of Assaf et al (1981) are overestimates, and thus that $Cl^-$ is less important in the depolarizing response of the dendrites than might be supposed otherwise. In FIG 3.5A, depolarizing responses of the dendrites to GABA at the same sites as in FIG 3.5B are shown. Here, the responses are the maximum responses that could be obtained to a given iontophoretic current. The small response obtained from dendrites near the fissure may demonstrate that the voltage size of the response was decrementally conducted to the cell body. These arguments assume that the reversal potential for the depolarizing responses to GABA at all the sites was the same.

The change in reversal potential produced low $Cl^-$ ACSF measured by displacement of the membrane potential of the cell body was 5mV, when $E_{Cl}$ was changed by 10mV. This suggests that the depolarizing response of the cell body was not entirely mediated by $Cl^-$.

4.2.5. EFFECT OF PICROTOXIN ON THE DEPOLARIZING RESPONSE OF THE DENDRITES TO GABA AND EDA.

The involvement of chloride channels in the depolarizing response of the dendrites to GABA and EDA was further tested by applying the drug picrotoxin. Picrotoxin reduced the depolarizing response of the dendrites to GABA or EDA, in some cases by up to 70% (FIG 3.28) but did not block the response completely.

This result is difficult to explain if the depolarizing response to GABA and EDA is mediated by more than one ion. For a purely $Cl^-$ mediated response, the interpretation is obvious; picrotoxin reduced the response as expected. For a mixed response, blocking the $Cl^-$ component would result in the reversal potential of the depolarizing response becoming less negative. This is because the reversal potential of the response lies somewhere between $E_{Cl}$ and $E_{Na}$. $E_{Cl}$ is closer to the membrane potential than $E_{Na}$. Whether $E_{Cl}$ is less negative or more negative than the membrane potential is not important for this argument. If the reversal potential of the depolarizing response was thus made less negative by picrotoxin, then the size of the depolarization would have increased. If the depolarizing response of the dendrites is mediated by both $Cl^-$ and $Na^+$ conductances, then it appears that picrotoxin is blocking both conductances. There was no effect of picrotoxin on the reversal potential. This argument is partly supported by Bowery and Brown (1974) who found that picrotoxin not only blocks a $Cl^-$ dependent response to GABA in autonomic ganglia (albeit at 50$\mu$M), but also reduced a $Na^+$

dependent response to carbachol (at 200μM). An effective concentration in the present study was 2μM.

It is possible that picrotoxin acted on some site on the GABA receptor complex that is not the chloride channel. Picrotoxin is unlikely to act directly on the GABA binding site as ligand binding studies have shown that it has no effect on the affinity of the site for GABA.

4.2.6. THE EFFECT OF IONS OTHER THAN SODIUM AND THE EFFECT OF PICROTOXIN ON THE HYPERPOLARIZING RESPONSES OF THE DENDRITES AND CELL BODY TO GABA AND EDA.

The hyperpolarizing response of the dendrites to EDA appeared to be $K^+$ dependent. The size of the response was unaffected by other ions and the reversal potential may have been at $E_K$. The hyperpolarizing response of the dendrites to GABA or EDA was unaffected by picrotoxin at concentrations which effectively reduced the depolarizing response of the dendrites. This latter is a similar result to that of Alger and Nicoll (1982), who used THIP as an agonist to obtain the hyperpolarization of the dendrites. If this hyperpolarization of the dendrites is the same as the physiological synaptic response, these observations may explain why picrotoxin has been reported to be ineffective in the hippocampus.

The hyperpolarizing response of the cell body appears to

be $K^+$ dependent although it is possible that the effects of low $Cl^-$ ACSF on the response cannot be explained entirely as interference from the depolarizing response of the nearby dendrites. Picrotoxin sometimes reduced this response, which may argue for an involvement of chloride channels in the hyperpolarizing response of the cell body to GABA or EDA.

### 4.2.7. IS THERE A CHLORIDE PUMP IN THE DENDRITES?

Since chloride ions are often thought to be distributed passively in excitable tissue, it was of interest to examine how a chloride gradient could be maintained in the dendrites. Ammonium ions block chloride pumps in spinal motoneurones (Lux, 1971). The pump in these neurones is outwardly directed, so $Cl^-$-mediated responses are hyperpolarizing. Ammonium ions had no significant effects in the present experiments, apart from causing irreversible depolarzation of the neurone.

Frusemide, which blocks $Cl^-$ readsorption in the kidney, had an effect in one experiment only. Frusemide shifted the reversal potential of the depolarizing response of the dendrites to GABA to a less negative level, an effect that is consistent with a blockade of $Cl^-$ uptake. Since the effect was observed on only one occasion, the result is not conclusive. At the highest concentrations used, SITS, which affects anion exchange mechanisms in various tissues, reduced the size of the depolarizing response of the dendrites to GABA, and may have had an effect on the uptake of $Cl^-$ into

dendrites that were depleted of $Cl^-$. This suggests that there is an active mechanism for $Cl^-$ uptake. This hypothesis could be tested by blocking metabolism of the slice or cooling the slice, although such manipulations might also affect the neurones generally.

4.2.8. THE BENZODIAZEPINES.

The depolarizing response of the dendrites to EDA or GABA was potentiated by water-soluble benzodiazepines. This suggests that the receptor mediating the response is the $GABA_A$ receptor. This is supported by the block of the depolarizing response of the dendrites by bicuculline. The depolarization was also sometimes reduced by the benzodiazepines, an effect of these drugs observed before (Biscoe, Duchen and Pascoe, 1983). The concentrations of benzodiazepines used in this study were very high compared with the therapeutic concentration. Assuming a volume of distribution of diazepam in man of 1 l/kg (Bowman and Rand, 1980) then the concentration in the brain is in the 100nM range, 10 to 100 times less than in these experiments. Such concentrations may have had antagonistic effects on the benzodiazepine receptor. It was interesting that flurazepam potentiated the hyperpolarizing response of the cell body to GABA, since this response appears to be $K^+$-dependent and not $Cl^-$-dependent; from binding studies, it appears that potentiation of binding of GABA by benzodiazepines is chloride dependent. This has often been taken to mean that a chloride

channel is involved in the GABA receptor complex. There is no direct evidence for such a hypothesis. The results of the present study do not show that there are different receptors for the different benzodiazepines.

# REFERENCES.

ACHERMAN, D and KUTSCHER, F. (1910).
Z. Physiol. Chem. 69 1265-1272. Cited in
GERSCHENFELD, A.M. 1973.

AICKIN, C.C. and BRADING, A.F. (1982). The effect of $CO_2/HCO_3$ and DIDS on Cl movements in guinea pig vas deferens.
Proc. Physiol. Soc. February. 97P-98P.

ALGER, B.E., JAHR, C.E. and NICOLL, R.A. (1981).
Electrophysiological analysis of GABAergic local circuit neurones in the central nervous system. pp77-91.
in 'GABA and Benzodiazepine receptors' eds COSTA, E., DI CHIARA, G and GESSA, G.L. Raven. New York.

ALGER, B.E. and NICOLL, R.A. (1979). GABA mediated biphasic inhibitory responses in the hippocampus.
Nature 281 315-317.

ALGER, B.E. and NICOLL, R.A. (1982). Pharmacological evidence for two kinds of GABA receptors on rat hippocampal pyramidal cells studied in vitro. J. Physiol.
328 125-141.

ALLAN, R.D., EVANS, R.H. and JOHNSTON, G.A.R. (1980).
Gamma-aminobutyric acid agonists; an in vitro comparison between depression of spinal synaptic activity and the depolarization of spinal root fibres in the rat. Brit. J. Pharmacol. 70 609-615.

ALLEN, G.I., ECCLES, J.C., NICOLL, R.A., OSHIMA, T. and RUBIA, F.J. (1977). The ionic mechanism concerned the in generating the IPSPs in hippocampal pyramidal cells. Proc. Roy. Soc. 198 363-384.

AMES, A., SAKANOVE, M. and ENDO, S. (1964). Na, K, Ca, Mg and Cl concentration in choroid plexus fluid and cisternal fluid compared to plasma ultrafiltrate. J. Neurophysiol. 27 672-681.

ANDERSEN, P. (1960). Interhippocampal inpulses. Acta Physiol. Scand. 48 178-208.

ANDERSEN, P., ECCLES, J.C. and LOYNING, Y. (1964). Location of postsynaptic inhibitory synapses on hippocampal pyramids. J. Neurophysiol. 27 592-607 and Pathway of postsynaptic inhibition in the hippocampus. J. Neurophysiol. 27 608-619.

ANDERSEN, P., BLISS, T.V.P. and SKREDE, K.K. (1971). Lamellar organisation of hippocampal excitatory pathways. Exp. Brain Res. 13 222-238.

ANDERSEN, P., DINGLEDINE, R., GJERSTAD, L., LANGMOEN, I.A. and MOSFELDT-LARSEN, A. (1980). Two different responses of hippocamapal pyramidal cells to application of GABA. J. Physiol. 305 279-296.

ANDERSEN, P. and LOMO, T. (1966). Mode of inactivation of hippocampal pyramidal cells by excitatory synapses on dendrites. Exp. Brain Res. 2 247-260.

ASANO, T and SPECTOR, S. (1979). Identification of inosine and hypoxanthine as endogenous ligands for brain benzodiazepine binding sites. Proc. Natl. Acad. Sci. USA 76 977-981.

ASSAF, S.Y., CRUNELLI, V. and KELLY, J.S. (1981). Electrophysiology of rat dentate gyrus in vitro. In 'Electrophysiology of isolated mammalian CNS preparations'. Eds KERKUT, G.A. and WHEAL, H.V. pp153-188. Academic Press. London.

ASSAF, S.Y, CRUNELLI, V. and KELLY, J.S. (1981b). Small amplitude spikes recorded intracellularly from the rat dentate gyrus. Proc. Physiol. Soc. March 45P.

AWAPARA, J., LANDUA, A.J., FUERST, R. and SEALE, B. (1950). Free gamma-amino butyric acid in the brain. J. Biol. Chem. 187 35-39.

BALAZS, R., HAJOS, F, JOHNSON, A.L., REYNIERSE, G.L.A., TAPIA, R. and WILKIN, G.P. (1975). Subcellular fractionation of rat cerebellum; an electron microscopic and biochemical investigation. Brain Res. 86 17-30.

BARKER, J.L. and NICOLL, R.A. (1973). The pharmacology and ionic dependency of amino acid responses in frog spinal cord. J. Physiol. 228 259-277.

BARKER, J.L. and RANSOM, B.R. (1978). Amino-acid pharmacology of mammalian central neurones grown in culture. J. Physiol. 280 1-24.

BARRETT, J.N. and CRILL, W.E. (1974). Specific membrane properties of cat motoneurones. J. Physiol. 301-324.

BAXTER, C. (1970). The nature of gamma-amino butyric acid. In 'Handbook of Neurochemistry' 3 pp289-353. Ed LAJTHA, A. Plenum Press. New York.

BISCOE, T.J., DUCHEN, M.R. and PASCOE, J.E. (1983). GABA/benzodiazepine interactions in the mouse hippocampal slice. Proc. Physiol. Soc. March 1983 13P.

BLACKSTAD, T.W. (1956). Commisural connections in the hippocampal region in the rat, with special reference to their mode of termination. J. comp. Neurol. 205 417-537.

BLAXTER, T.J. and COTTRELL, G.A. (1982). Responses of rat hippocampal pyramidal cells to GABA and ethylenediamine. J. Physiol. 330 46P.

BLAXTER, T.J. (1983). Storing brain slices. J. Neurosci. Meths. 7 397-398.

BOND, P.A. (1973). The uptake of $^{3}$H-GABA by slices of various regions of the brain and the effect of lithium. J. Neurochem. 20 511-517.

BOISTEL, J. and FATT, P. (1958). Membrane permeability changes during inhibitory transmitter action in crustacean muscle. J. Physiol. 144 176-191.

BORMANN, J. SAKMANN,B. and SEIFERT, W. (1983). Isolation of GABA-activated single channel $Cl^{-}$ currents in the soma membrane of rat hippocampal neurones. Proc. Physiol. Soc. March 1983 14P.

BOWERY, N.G. and BROWN, D.A. (1974). Depolarizing action of gamma-amino butyric acid and related compounds on rat superior cervical ganglia *in vitro*. Brit. J. Pharmacol. 50 205-219.

BOWERY, N.G., DOBLE, A., HILL, D.R., HUDSON, A.L., SHAW, J. and TURNBULL, M.J. (1980). β-chlorophenyl GABA is a selective ligand for a novel GABA receptor on nerve terminals. Brain Res. Bull. 5 supplement 2 497-502.

BOWERY, N.G., HILL, D.R., HUDSON, A.L., PERKINS, M.N. and STONE, T.W. (1983). GABA receptor binding in physiological salt solution. Proc. Brit. Pharmacol. Soc. April. C86.

BOWMAN, W.C. and RAND, M.J. (1980). Textbook of Pharmacology. Chpt 40 p19. Blackwell.

BRAESTRUP, C., ALBRECHTSON, R. and SQUIRES, R. (1977). High densities of benzodiazepine receptors in human brain. Nature 269 702-704.

BRAESTRUP, C. and NIELSEN, M. (1981). $^{3}$H-propyl β-carboline-3-carboxylate as a selective radioligand for $BZ_1$ benzodiazepine receptor subclass. J. Neurochem. 37 333-341.

BRAESTRUP, C., NIELSEN, M. and OLSEN, C.E. (1980). Urinary and brain β-carboline-3-carboxylate as potent inhibitors of brain benzodiazepine receptors. Proc. Natl. Acad. Sci. USA 77 2288-2292.

BRINLEY, F.J., KANDEL, E.R. and MARSHALL, W.H. (1960). Effect of GABA on K42 outflow from rabbit cortex. J Neurophysiol. 23 377.

BROGDEN, R.N., SPEIGHT, T.M. and AVERY, G.S. (1974). Baclofen; a preliminary report of its pharmaclogical properties and therapeutic efficacy in spasicity. Drugs 8 1.

BROWN, D.A. and SCHOLFIELD, C.N. (1979). Depolarization of neurones in the isolated olfactory cortex of the guinea pig by gamma aminobutyric acid. Brit. J. Pharmacol. 65 339-345.

BROWN, T.H., FRICKE, R.A. and PERKEL, D.H. (1981). Passive electrical constants in three classes of hippocampal neurones. J. Neurophysiol. 46 812-827.

CHIU, T.H. and ROSENBERG, H.C. (1983). Conformation changes in benzodiazepine receptors induced by the antagonist Rol5-1788. Mol. Pharmacol. 23 289-294.

COBBETT, P. (1980). A novel 3-way tap. J. Neurosci. Meths. 3 101-104.

COBBETT, P. and COTTRELL, G.A. (1981). The structural integrity of neurones in hippocampal slice preparation as revealed by intracellular injection of Lucufer Yellow. J. Neurocytol. 10 671-678.

CONSTANTI, A. (1978). A mixed effect of picrotoxin on GABA dose/conductance relations recorded in lobster muscle. Neuropharmacol. 17 159-167.

CONSTANTI, A and NISTRI, A. (1976). A comparative study of the action of GABA and piperazine on the lobster muscle fibre and frog spinal cord. Brit. J. Pharmacol. 57 347-358.

COOMBS, J.G., ECCLES, J.C. and FATT, P. (1955). The specific ion conductances and ionic movements across the motoneuronal membrane that produce the inhibitory post-synaptic potential. J. Physiol. 130 326-373.

CRAGG, B.G. and HAMLYN, L.H. (1955). Action potential of the pyramidal neurones in hippocampus of the rabbit. J. Physiol. 129 608-627.

CURTIS, D.R., DUGGAN, A.W. and FELIX, D. (1970). GABA and the inhibiton of Deiter's nucleus. Brain Res. 23 117-120.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. and JOHNSTON, G.A.R. (1970). GABA, bicuculline and central inhibition. Nature 226 1222-1224.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. and JOHNSTON, G.A.R. (1971). Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. Brain Res. 32 69-96.

CURTIS, D.R., DUGGAN, A.W., FELIX, D., JOHNSTON, G.A.R. and MCLENNAN, H. (1971). Antagonism between bicuculline and GABA in cat brain. Brain Res. 33 57-73.

CURTIS, D.R., FELIX, D. and MCLENNAN, H. (1970). GABA and hippocampal inhibition. Brit. J. Pharmacol. 40 881-883.

CURTIS, D.R. and TEBECIS, A.K. (1972). Bicuculline and thalamic inhibition. Exp. Brain Res. 16 210-218.

DE GROAT, W.C., LALLEY, P.M. and SAUM, W.R. (1972). Depolarization of dorsal root ganglia in the cat by GABA and related amino acids; antagonism by picrotoxin and bicuculline. Brain Res. 44 273-277.

ECCLES, J.C. (1957). The physiology of nerve cells. Baltimore. John Hopkins Press. 270pp.

ECCLES, J.C., SCHMIDT, R.F. and WILLIS, W.D. (1963). Pharmacological studies on presynaptic inhibition. J. Physiol. 168 500-530.

FORSTER, P., LLOYD, H.G.E., MORGAN, P.F., PARKER, M., PERKINS, M.N. and STONE, T.W. (1981). Ethylenediamine acts upon GABA receptors and uptake sites. Brit. J. Pharmacol. 74 274P.

FUJIMOTO, M. and OKABAYASHI, T. (1981). Effect of picrotoxin on benzodiazepine receptors and GABA receptors with reference to the effect of chloride ion. Life Sci. 28 895-901.

GALLAGHER, D.W. (1978). Benzodiazepines; potentiation of GABA inhibitory response in dorsal raphe nucleus. Euro. J. Pharmacol. 49 133-143.

GALLAGHER, D.W., HIGASHI, S. and NISHI, S. (1978). Characterization and ionic basis of GABA induced depolarizations recorded in vitro from cat primary afferent neurones. J Physiol. 275 263-282.

GINSBORG, B.L. (1967). Ion movements in junctional transmission. Pharmac. Revs. 19 289-315.

GRUNDFEST, H., REUBEN, J.P. and RICKLES, W.H. (1959). The electrophysiology and pharmacology of lobster neuromuscular synapses. J. Gen. Physiol. 42 1301-1323.

GRUOL, D.L., BARKER, J.L., HUANG, L.Y.M., MACDONALD, J.F. and SMITH, T.G. (1980). Hydrogen ions have multiple effects on the excitability of cultured mammalian neurones. Brain Res. 183 247-252.

GUTNICK, M.J. and SEGAL, M. (1981). Serotonin and GABA induced fluctuations in extracellular ion concentrations in the hippocampal slice. pp261-265. In 'Ion selective microelectrodes and their use in excitable tissue'. Eds SYKOVA, E., HNIK, P. and VYKLICKY, L. Plenum. New York.

HAAS, H.L., SCHAERER, B. and VOSMANSKY, M. (1979). A simple perfusion chamber for the study of nervous tissue slices in vitro. J. Neurosci. Meths. 1 322-325.

HAAS, H.L. and KONNERTH, A. (1983). Histamine and noradrenaline reduce calcium-activated potassium conductance in hippocampal pyramidal cells. Nature 302 432-434.

HAGIWARA, S., KUSANO, K. and SAITO, S. (1960). Membrane changes in crayfish stretch receptor neurone during synaptic activity and under the action of gamma-amino butyric acid. J. Neurophysiol. 23 505-515.

HALL, Z.W., BOWNDS, M.D. and KRAVITZ, E.A. (1970). The metabolism of gamma-amino butyric acid in the lobster nervous system. Enzymes in single excitatory and inhibitory axons. J. Cell Biol. 46 290-299.

HEATH, R.G. and HARPER, J.W. (1974). Ascending projection of the cerebellar nucleus fastigii to the hippocampus, amygdala and other temporal lobe sites. Evoked potential and histological studies in monkeys and cats. Exp. Neurol. 45 268-287.

HILL, D.R. (1983). The GABA-mimetic activity of EDA is dependent on physiological concentrations of $HCO_3$. Proc. Brit. Pharmacol. Soc. April C109.

HIRSCH, J.D., KOCHMAN, R.L. and SUMNER, P.R. (1982). Heterogeneity of brain benzodiazepine receptors demonstrated by $^3$H-propyl β-carboline-3-caboxylate binding. Mol. Pharmac. 21 618-628.

HOKFELT, T. and LJUNGDAHL, A. (1972). Autoradiographic identification of cerebellar and cerebral cortical neurons accumulating labelled GABA. Exp. Brain Res. 14 354-362.

HOMMA, S. and ROVAINEN, C.M. (1978). Conductance increases produced by glycine and GABA in lamprey neurones. J. Physiol. 279 231-252.

ITO, M and YOSHIDA, M. (1966). The origin of cerebellar induced inhibition of Deiter's neurones. Exp. Brain Res. 2 330-349.

IVERSEN, L.L. and NEAL, M.J. (1968) The uptake of $^{3}$H-GABA by slices of cerebral cortex. J. Neurochem. 15 1141-1149.

IVERSEN, L.L. and KRAVITZ, E.A. (1968). The metabolism of GABA in the lobster nervous system: uptake of GABA in nerve-muscle preparations. J. Neurochem. 15 609-620.

IVERSEN, L.L. and SCHON, F.F. (1973). The use of autoradiographic techniques for identification and mapping of transmitter-specific neurones in CNS. In 'New concepts in neurotransmitter regulation' pp153-193 Ed MANDEL, A.J. Plenum. New York.

JAHNSEN, H. and MOSFELT LARSEN A.M. (1981). The effects of a benzodiazepine on the hyperpolarizing and depolarizing responses of hippocampal cells to GABA. Brain Res. 207 214-217.

JOHNSTON, D. (1981). Passive cable properties of hippocampal CA3 pyramidal cells. Cell. Mol. Neurobiol. 1 41-55.

KANDEL, E.R., SPENCER, W.A. and BRINLEY, F.J. (1961). Electrophysiology of hippocampal neurones. J. Neurophysiol. 24 225-285.

KAROBATH, M., DREXLER, G. and SUPAVILAI, P. (1981). Modulation by picrotoxin and IPBTO of flunitrazepam binding to the GABA-benzodiazepine receptor complex of the rat cerebellum. Life Sci. 28 307-313.

KRNJEVIC, K. and SCHWARTZ, S. (1967). The action of GABA on cortical neurones. Exp. Brain Res. 3 320-326.

KUDO, Y. and FUKUDA, H. (1976). Alteration of extracellular $K^+$ activity induced by amino acids in frog spinal cord. Jpn. J. Pharmacol. 26 385.

LANGMOEN, I.A., ANDERSEN, P., GJERSTAD, L., MOSFELDT LARSEN, A. and GANES, T. (1978). Two separate effects of GABA on hippocampal pyramidal cells in vitro. Acta Physiol. Scand. 102 28-29.

LANGMOEN, I.A. and ANDERSEN, P. (1981). The hippocampal slice in vitro. In 'Electrophysiology of Isolated Mammalian CNS Preparations' pp153-188. Eds KERKUT, G.A. and WHEAL, H.V. Academic Press. London.

LEEB-LUNDBERG, F. and SNOWMAN, A. (1981). Perturbation of benzodiazepine receptor binding by pyrazolopyridazines involves picrotoxin/ barbiturate receptor sites. J. Neurosci 1 471-477.

LEEB-LUNDBERG, F. and OLSEN, R.W. (1981). Interactions of barbiturates of various pharmacological categories with benzodiazepine receptors. Mol. Pharmac. 21 320-328.

LEGGE, K.F., RANDIC, M. and STRAUGHAN, D.W. (1966). The pharmacology of neurones in the pyriform cortex. Brit. J. Pharmacol. Chemother. 26 87-107.

LLINAS, R., BAKER, R. and PRECHT, W. (1974). Blockade of inhibition by ammonium acetate; action on chloride pump in cat trochlear motoneurones. J. Neurophysiol. 37 522-532.

LLOYD, H.G.E., PERKINS, M.N. and STONE, T.W. (1982) Ethylenediamine as a specific releasing agent of GABA in rat striatal slices. J. Neurochem. 38 1168-1169.

LO, M., STRITTMATER, S.M. and SNYDER, S.H. (1982). Physical separation of and characterization of two types of benzodiazepine receptor. Proc. Natl. Acad. Sci. USA 79 680-684.

LORENTE DE NO, R. (1934). Studies on the structure of the cerebral cortex. ii. Continuation of the study of the ammonic system. J. Psychol. Neurol. Leipzig 46 113-177.

LUX, H.D. (1971) Ammonium and chloride extrusion: hyperpolarizing synaptic inhibition in spinal motoneurons. Science 173 555-557.

MACDONALD, R. and BARKER, J.L. (1977). Nature 271 563-564.

MAURER, R. (1979). THIP does not interfere with the benzodiazepine binding site on rat cortical membranes. Neurosci. Letts. 12 65-68.

MORGAN, P.F., PERKINS, M.N. and STONE, T.W. (1982). Ethylenediamine potentiates *in vitro* $^{3}$H-diazepam binding to rat cerebral cortex. Brit. J. Pharmac. 75 95P.

MOTOKIZAWA, F., REUBEN, J.P. and GRUNDFEST, H. (1969). Ionic permeability of inhibitory post-synaptic membrane of lobster muscle fibres. J. Gen. Physiol. 54 437-461.

NESTOROS, J.N. (1980) Electrophysiological evidence that GABA potentiation *in vivo* correlates with benzodiazepine receptor affinity. Brain Res. Bull. 5 supplement 2 849-852.

NESTOROS, J.N. and NISTRI, A. (1979) Effects of microiontophoretically applied flurazepam on responses of cerebral cortical neurones to putative neurotansmitters. Can. J. Physiol. Pharmacol. 57 1324-1329.

NICOLL, R.A. (1971). Pharmacological evidence for GABA as a transmitter in granule cell inhibition in the olfactory bulb. Brain Res. 35 137-149.

NIELSEN, M. and BRAESTRUP, C. (1980). Ethyl-β-carboline carboxylate shows differential benzodiazepine receptor interaction. Nature 286 606-607.

NISHI, S., MINOTA, S. and KARCZMAR, A.G. (1974). Primary afferent neurones; the ionic mechanism of GABA mediated depolarization. Neuropharmac. 13 215-219.

OBATA, K. (1976). Excitatory effects of GABA. In ´ GABA in CNS function´. Eds. ROBERTS, E., CHASE, T.N. and TOWER, D.B. Raven.

OBATA, K. and HIGHSTEIN, S.M. (1970). Blocking by picrtoxin of both vestibular inhibition and GABA action on rabbit occulomotor neurones. Brain Res. 18 538-541.

OBATA, K., ITO, K., OCHI, R. and SATO, N. (1967). Pharmacological properties of the post-synaptic inhibition by Purkinje cell axons and the action of GABA on Deiter´s neurones. Exp. Brain Res. 4 43-57.

OBATA, K. OTSUKA, M. and TANAKA, Y. (1970). Determination of GABA in single nerve cells of cat CNS. J. Neurochem. 17 697-698.

OBATA, K. and TAKEDA, K. (1969). Release of GABA into the fourth ventricle induced by stimulation of the cat´s cerebellum. J. Neurochem. 16 1043-1047.

OLSEN, R.W. (1982). Drug interactions at the GABA receptor ionophore complex. Ann. Rev. Pharmacol. Toxicol. 22 245-277.

OKAMOTO, K. and SAKAI, Y. (1979). Augmentation by chlordiazepoxide of the inhibitory effects of taurine, β-alanine and GABA on spike discharges in guinea pig cerebellar slices. Brit. J. Pharmacol. 65 277-285.

ORKAND ,P. and KRAVITZ, E.A. (1971). Localization of the sites of gamma-amino acid uptake in lobster nerve-mucsle preparations. J. Cell Biol. 49 73-89.

OTSUKA, M., IVERSEN, L.L., HALL, Z.W. and KRAVITZ, E.A. (1966). Release of gamma-amino butyric acid from inhibitory nerves of lobster. Proc. Natl. Acad. Sci. 56 1110-1115.

PATEL, A.J., BALAZS, R. and RICHTER, D. (1970). Contribution of the GABA bypath to glucose oxidation. Nature 226 1160-1161.

PAUL, S.M., MARANGOS, P.J. and SKOLNICK, P. (1981). The benzodiazepine-GABA-chloride ionophore receptor complex: common site of minor tranquilliser action. Biological Psychiatry 16 213-229.

PERKINS, M.N., BOWERY, N.G., HILL, D.R. and STONE, T.W. (1981). Neuronal responses to ethylenediamine; preferential blockade by bicuculline. Neurosci. Letts. 23 325-327.

PERKINS, M.N. and STONE, T.W. (1982). Comparison of the effects of ethylenediamine analogues and gamma amino butyric acid on cortical and pallidal neurones. Brit. J. Pharmacol. 75 93-99.

PURPURA, D.P., MCMURTY, J.G., LEONARD, C.F. and MALLIANI, A. (1966). Evidence for the dendritic of spikes without depolarizing prepotentials in hippocampal neurones during and after seizure. J. Neurophysiol. 29 954-979.

PURVES, R.D. (1981). ´Microelectrode methods for intracellular recording and iontophoresis.´ p16. Academic Press. London.

RALL, W. (1959). Branching dendritic trees and motoneurone membrane resistivity. Exp. Neurol. 1 491-529.

REGAN, J.W., ROESKE, W.R., MALICK, J.B., YAMAMURA, S.H. and YAMAMURA, H.I. (1981). GABA enhancement of CL218,872 affinity and evidence of benzodiazepine receptor heterogeneity. Mol. Pharmacol. 20 477-483.

RICHARDS, C.D. and SERCOMBE, R. (1970). Calcium, magnesium and the electrical activity of guinea-pig olfactory cortex *in vitro*. J. Physiol. 211 571-584.

SCHOLFIELD, C.N. (1978). A depolarizing inhibitory potential in neurones of the olfactory cortex *in vitro*. J. Physiol. 275 547-557.

SCHWARTZKROIN, P. (1975). Characteristics of CA1 neurones recorded intracellularly in the hippocampal *in vitro* slice preparation. Brain Res. 85 423-436.

SEGAL, M., SIMS, K. and SMISSMAN, E. (1975). Characterization of an inhibitory receptor in rat hippocampus. Brit. J. Pharmacol. 54 181-188

SIEGHART, W. and KAROBATH, M. (1980). Molecular heterogeneity of benzodiazepine receptors. Nature 286 285-287.

SIMMONDS, M.A. (1980). Evidence that bicuculline and picrotoxin act at different sites to antagonize $\gamma$-amino butyric acid in rat cuneate nucleus. Neuropharmac. 19 39-45.

SQUIRES, R.F., BENSON, D.I., BRAESTRUP, C., COUPET, J., KLEPNER, C.A., MYERS, V. and BEER, B. (1979). Some properties of brain-specific benzodiazepine receptors: new evidence for multiple receptors. Pharmacol. Biochem. Behav. 10 825-830.

STEINER. F.A. and FELIX, D. (1976). Antagonistic effects of GABA and benzodiazepines on vestibular and cerebellar neurones. Nature 260 346-347.

STEINER, F.A. and HUMMEL, P. (1968). Effects of nitrazepam and phenobarbital on hippocampal and lateral geniculate neurones in the cat. Int. J. Neuropharmacol. 7 61-69.

STORM-MATHIESON, J. (1977). Localization of transmitter candidates in the brain; the hippocampal formation as a model. Prog. Neurobiol. 8 119-181.

STORM-MATHIESON, J., LEKNES, A.K., BORA, A.T., VAALAND, J.L., EDMINSON, P., HUANG, F.M.S. and OTTERSEN, O.P. (1983). First visualization of glutamate and GABA in neurones by immunocytochemistry. Nature 301 517-520.

SUPAVILAI, P. and KAROBATH, M. (1980). Benzodiazepine receptor heterogeneity in the cerebellum and the hippocampus. Eur. J. Pharmacol. 64 91-93.

SYTINSKY, I.A., SOLDATENKOV, A.T. and LJATHA, A. (1979). Neurochemical basis of the therapeutic effects gamma-aminobutyric acid and its derivatives. Prog. Neurobiol. 10 89-133.

TAKEUCHI, A. and TAKEUCHI, N. (1965). Localized action of gamma-amino butyric acid on crayfish muscle. J. Physiol. 177 225-238.

TAKEUCHI, A. and TAKEUCHI, N. (1969). A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. J. Physiol. 205 377-391.

TALLMAN, J.F. (1978). GABAergic modulation of benzodiazepine binding site sensitivity. Nature 274 383-385.

TALLMAN, J.F. (1980). Interactions between GABA and benzodiazepines. Brain Res. Bull. 5 supplement 2 829-832.

TRAUB, R.P. and LLINAS, R. (1979). Hippocampal pyramidal cells; significance of dendritic ionic conductances for neuronal function and epileptogenesis. J. Neurophysiol. 42 476-496.

TSUCHIYA, T. and FUKUSHIMA, H. (1978). Effects of benzodiazepines and pentobarbitone on the GABA-ergic recurrent inhibition of hippocampal neurones. Eur. J. Pharmacol. 48 421-424.

TURNER, D.A. and SCHWARTZKROIN, P.A. (1980). Steady state electrotonic analysis of intracellularly stained hippocamapal neurones. J. Neurophysiol. 44 184-199.

VAN DER KLOOT, W., ROBBINS, J. and COOKE, I. (1958). Blocking by picrotoxin of peripheral inhibition in the crayfish. Science 127 521-522.

WILLIAMS, J.T., EGAN, T.M. and NORTH, R.A. (1982). Encephalin opens $K^+$ channels in mammalian central neurones. Nature 299 74-77.

WONG, R.K.S. and PRINCE, D.A. (1979). Intradendritic recordings in hippocampal pyramidal neurones. Proc. Natl. Acad. Sci. USA. 76 986-990.

YOUNG, W.S., NIEHOFF, D., KUHAR, M.J., BEER, B. and LIPPA, A.S. (1981). Multiple benzodiazepine receptor localization by light microscope radiohistochemistry. J. Pharmacol. Exp. Therap. 216 425-430.